Institute for Molecular Medicine Finland (FIMM)
Helsinki Institute of Life Science (HiLiFE)
Doctoral School in Health Sciences (DSHealth)
Doctoral Programme in Biomedicine (DPBM)
University of Helsinki

STUDIES ON DRUG RESISTANCE AND MOLECULAR BIOMARKERS IN ACUTE MYELOID LEUKEMIA

Riikka Karjalainen

ACADEMIC DISSERTATION

Doctoral dissertation, to be presented for public discussion with the permission of the Faculty of Medicine of the University of Helsinki, in Porthania, lecture room PIII, Yliopistonkatu 3, Helsinki, on the 4th of October, 2019, at 12 noon.

Helsinki 2019

Supervisors

Dr. Caroline Heckman, Ph.D.
Institute for Molecular Medicine Finland
Helsinki Institute of Life Science
University of Helsinki
Helsinki, Finland

Professor Jonathan Knowles, Ph.D.
Institute for Molecular Medicine Finland
Helsinki Institute of Life Science
University of Helsinki
Helsinki, Finland

Reviewers

Adjunct Professor Urpu Salmenniemi, M.D., Ph.D.
Hematology and stem cell transplantation unit
Turku University Hospital
Turku, Finland

Adjunct Professor Matthias Nees, Ph.D.
Institute of Biomedicine
University of Turku
Turku, Finland

Opponent

Dr. Eva Szegezdi, Ph.D.
National University of Ireland
Galway, Ireland

ISBN 978-951-51-5380-7 (paperback)
ISBN 978-951-51-5381-4 (PDF)
Unigrafia
Helsinki 2019

The Faculty of Medicine uses the Urkund system (plagiarism recognition) to examine all doctoral dissertations.

“Nothing in life is to be feared. It is only to be understood”
Marie Curie

“What we know is a drop, what we don't know is an ocean”
Isaac Newton

To my family

TABLE OF CONTENTS

ABBREVIATIONS

ABC	ATP binding cassette
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
AUC	Area under the curve
BM	Bone marrow
BCL-2	B-cell chronic lymphocytic leukemia/lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCR	Breakpoint cluster region
BET	Bromodomain and extraterminal
BH3	BCL2 homology domain 3
CAR	Chimeric antigen receptor
CBF	Core-binding factor
CLL	Chronic lymhocytic leukemia
CLP	Common lymphoid progenitor
CM	Conditioned medium
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CR	Complete remission
CRi	Complete remission with incomplete blood recovery
CSF	Colony stimulating factor
CTG	CellTiter Glo
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSRT	Drug sensitivity and resistance testing
DSS	Drug sensitivity score
ELN	European Leukemia Net
EMA	European Medicines Agency
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FGF	Fibroblast growth factor
FLT3	Fms-like tyrosine kinase 3

G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GMP	Granulocyte/macrophage progenitor
GO	Gemtuzumab ozogamicin
HDAC	Histone deacetylation
HOX	Homeobox
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplant
JAK	Janus-associated kinase
IL	Interleukin
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia sequence 1
MCM	Mononuclear cell medium
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte/erythroid progenitors
MNC	Mononuclear cell
MOMP	Mitochondrial outer membrane permeabilization
MRD	Minimal residual disease
MSC	Mesenchymal stem cell
NCCN	National Comprehensive Cancer Network
NGS	Next generation sequencing
NSG	Non-obese diabetic/severe combined immunodeficient
PB	Peripheral blood
PCR	Polymerase chain reaction
PD1	Programmed cell death protein-1
PI3	Phosphatidylinositol 3
ROTS	Reproducibility optimized test statistic
siRNA	Small interfering RNA
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TKI	Tyrosine kinase inhibitor
WHO	World Health Organization
ZIP	Zero Interaction Potency

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Karjalainen R**, Pemovska T, Popa M, Liu M, Javarappa KK, Majumder MM, Yadav B, Tamborero D, Tang J, Bychkov D, Kontro M, Parsons A, Suvela M, Mayoral Safont M, Porkka K, Aittokallio T, Kallioniemi O, McCormack E, Gjertsen BT, Wennerberg K, Knowles J and Heckman C. JAK1/2 and BCL2 inhibitors synergize to counteract bone marrow stromal-cell induced protection of AML. *Blood*. 2017; 130:789 – 802.

II. **Karjalainen R***, Liu M*, Kumar A, Parsons A, He L, Malani D, Kontro M, Kallioniemi O, Porkka K and Heckman CA. Elevated expression of *S100A8* and *S100A9* correlates with resistance to the BCL-2 inhibitor venetoclax. *Leukemia*. 2019 (Epub ahead of print)

III. Elo LL, **Karjalainen R**, Ohman T, Hintsanen P, Nyman TA, Heckman CA and Aittokallio T. Statistical detection of quantitative protein biomarkers provides insights into signaling networks de-regulated in acute myeloid leukemia. *Proteomics*. 2014; 14:2443 – 2453.

* These authors contributed equally to this work

ABSTRACT

Acute myeloid leukemia (AML) is a clonal disease of immature hematopoietic cells. Treatment of AML patients is based on conventional chemotherapy and stem cell transplantation, but the majority of patients still suffer from relapse and poor overall survival. A growing body of evidence suggests that the bone marrow (BM) microenvironment plays an important role in protecting leukemic cells from drug-induced apoptosis, which consequently leads to accumulation of residual leukemic cells and eventual relapse. Although several factors are involved in leukemic cell-BM interactions, the exact mechanisms of these interactions and comprehensive knowledge of their impact on the activity of different drug classes is lacking. Better understanding of the molecular mechanisms of drug resistance could facilitate the development of improved treatment strategies, and means to monitor patients who are at risk for developing drug resistance. Modern technologies, such as RNA sequencing and proteomics, are widening our possibilities to learn more about the molecular basis of AML and to discover predictive biomarkers for therapy resistance.

In study I, we evaluated the effect of stromal cell secreted soluble factors on *ex vivo* drug responses in AML. We comprehensively evaluated how mononuclear cells (MNCs) collected from AML patients respond to 304 different inhibitors in stromal cell-conditioned medium (CM) compared to standard cell culture medium. From this study, we discovered that the stroma-derived factors altered response to 12% (36/304) of the drugs. Amongst the drugs, sensitivity to BCL-2 inhibitor venetoclax was significantly reduced by the stromal conditions. In follow-up experiments, we found that this effect could be overcome by inhibition of activated JAK/STAT signaling.

In study II, we investigated gene expression profiles that were associated with resistance to BCL-2 inhibitor venetoclax. *Ex vivo* and *in vitro* analyses of AML showed that high expression of *S100A8* and *S100A9* calcium binding family genes correlates positively with venetoclax resistance. In contrast, BET (bromodomain and extraterminal) inhibitor OTX-015 acted synergistically with venetoclax in resistant AML cell lines and patient samples.

In study III, our aim was to determine the applicability of proteins in biomarker discovery. We compared marker panels from proteomic and microarray

transcriptomic assays using reproducibility optimized test statistic (ROTS) in the context of AML dysregulated processes and networks. The analysis led to discovery of protein markers specific for AML using LC-MS/MS derived data.

In summary, this thesis shows that JAK/STAT inhibitors can counteract BM stroma-mediated resistance to the BCL-2 inhibitor venetoclax. Furthermore, we discovered a gene expression profile that correlates with *ex vivo* venetoclax resistance in AML and provide evidence of AML-related biomarkers from a proteomics dataset.

1 INTRODUCTION

AML is a complex disease with a relatively small number of recurrent mutated genes compared to other cancers[1,2]. Improved understanding of AML pathobiology has led to advances in the development of targeted therapies specific for disease-causing mutations with less toxic treatment options now available for AML patients. Despite the on-going revolution in AML therapy, development of drug resistance remains a key challenge for accomplishing long-term responses and possibly curing the disease[3]. Due to the remarkable plasticity, AML cells are capable of evading toxic effects of drugs through various escape routes that harness the body's normal control mechanisms[4].

The interplay between AML cells and the BM microenvironment has been recognized as an important contributor to disease progression[5]. However, knowledge of the molecular mechanisms of this interaction in relation to therapy resistance remains inadequate, especially regarding new targeted therapies. Moreover, new biomarkers are needed for identifying patients who would benefit from small-molecule inhibitors with novel mechanisms of action beyond those inspired by genomics, such as B-cell lymphoma (BCL-2) inhibitors[6]. As new drugs make their way to the clinics and change AML treatment, it is important to discover indicators of sensitivity and primary resistance. Biomarkers could be used to design novel treatment strategies that can possibly overcome drug resistance and lead to permanent cures.

In this thesis, our aim was to characterize tumor-stroma interactions, to identify predictors of *ex vivo* drug resistance, and to discover protein level markers for deregulated signaling networks in AML. Furthermore, high-throughput drug sensitivity and resistance testing was used to find drug combinations that could overcome stroma-induced drug resistance of AML patient samples. By integrating transcriptomic and *ex vivo* drug sensitivity data from AML patients, we identified biomarkers for BCL-2 inhibitor resistance. Finally, we evaluated the value of protein markers for AML biomarker discovery.

2 REVIEW OF THE LITERATURE

2.1 Hematopoiesis

Hematopoiesis is a tightly coordinated process, which generates over 100 billion blood cells every day[7,8]. The generated cells are made from a small pool of hematopoietic stem cells (HSCs) with the capacity to self-renew and differentiate.[9-12] During hematopoiesis, HSCs lose their self-renewal capacity whilst giving rise to common progenitors of myeloid (CMP) or lymphoid lineage (CLP).[13-15] Myeloid progenitors further differentiate into megakaryocyte/erythroid progenitors (MEPs) or granulocyte/macrophage progenitors (GMPs)[14] through lineage commitment. MEPs form platelets and erythrocytes, while GMPs give rise to neutrophilic, eosinophilic, and basophilic granulocytes and monocytes/macrophages. CLPs give rise to cells of the lymphoid lineage including B and T lymphocytes, natural killer cells and dendritic cells[15] (Figure 1). Mature blood cells carry out vital functions in the body including immunity, tissue remodeling and oxygen transport.

Several transcription factors regulate HSC formation and subsequent lineage-commitment. The most important factors for HSC production are Runx1, SCL/tal1, LMO2 and GATA-2, whereas HSC self-renewal is controlled by Tel, Bmi-1 and Gfi-1. Importantly, specific AML-associated translocations are known to affect these transcription factors including *TEL-RUNX1 t(12;21)*[16]. Lineage commitment is influenced by transcription factors that promote specific lineage-switches and factors that antagonize paralleling lineage[17]. As an example, *GATA-1* expression drives the erythroid/megakaryocyte differentiation from CMPs, and in parallel interferes with the *PU.1* promoted granulocyte/macrophage lineage[18]. Full erythroid/megakaryocyte development is accomplished with the expression of *FOG-1*, a cofactor of *GATA-1*[19,20], whereas *C/EBP* together with *PU.1* are important for myeloid differentiation[21,22]. Other key regulators of hematopoiesis, Ikaros and Pax-5, play a role in lymphoid[23,24] and B-cell development[25,26], respectively.

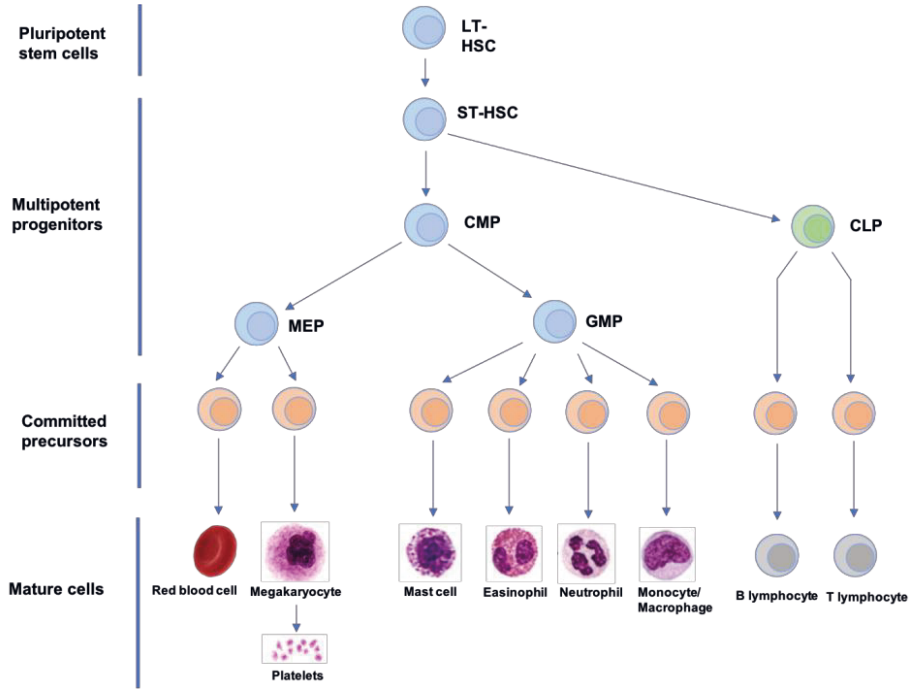

Figure 1. Hematopoietic cell differentiation. Long-term hematopoietic stem cell, LT-HSC; Short-term hematopoietic stem cell, ST-HSC; common myeloid progenitor, CMP; common lymphoid progenitor, CLP; megakaryocyte/erythroid progenitor, MEP; granulocyte/macrophage progenitor, GMP. *Adapted from Orkin et al, Cell 2008*[17].

2.1.1 Regulation of hematopoiesis

2.1.1.1 Bone marrow microenvironment

HSCs reside in the BM niche, which governs their differentiation and quiescence.[27,28] The BM niche is a unique milieu composed of various stromal cell populations, extracellular matrix, and a tight network of vasculature. The area is lined by osteoblasts that separate the marrow from mineralized bone[28,29]. Looking deeper into the cellular composition, we see that BM stromal cells are a mixture of cells that originate from mesenchymal stem cells (MSCs). These cells include osteoblasts, chondrocytes, adipocytes, fibroblasts and perivascular stromal cells including CXCL12-abundant reticular cells. Moreover, endothelial cells,

macrophages and nervous cells are considered as additional components of the BM stroma. The stromal cells secrete distinct growth factors and extracellular molecules such as glycosaminoglycans, glycoproteins and collagen that form the extracellular matrix[30]. The majority of HSCs exist in the highly vascularized endosteal region of the BM, in close proximity to sinusoidal vessels, which arise from arterioles[31]. The sinusoids form a reticular network through which mature blood cells are released to the systemic circulation.[32]

HSCs are mobilized and retained in the BM through interactions with adhesion molecules such as integrins (VLA-4 and VLA-5)[33], laminins[34], CD44[35], E-selectins[36,37], CXCL12[38], immunoglobulin-like receptors[39] and osteopontin[40]. Retention to BM depends on several cytokines, especially CXCL12 (or stromal-derived factor 1, SDF-1) that binds to its receptor CXCR4 on HSCs.[41,42] HSCs can be mobilized from the BM by targeting these interactions either with a CXCR4 inhibitor (plerixafor) or by reducing the number of CXCL12 expressing stromal cells with growth factors such as granulocyte-colony stimulating factor (G-CSF).

Interestingly, the sympathetic nervous system has also been reported to play part in HSC release from the BM through nerve fibers that form synapses on perivascular cells[37]. Apparently, the neural stimulus creates important circadian regulation on CXCR4 and CXCL12 expression and circulation of HSCs[43]. Non-myelinating Schwann cells wrapping the sympathetic nerves have been described to control HSC quiescence by secreting transforming growth factor-β (TGF-β)[44]. Other cell types contributing to BM niche regulation include adipocytes, megakaryocytes, macrophages and osteolineage cells. Contrary to earlier reports, the effect of osteolineage cells on HSC function appears to be indirect, as deletion of key HSC regulatory genes from osteoblasts was seen to have a minimal impact on their function[41,45].

2.1.1.2 Hematopoietic growth factors and signal transduction

Hematopoietic growth factors are glycoprotein hormones that sustain hematopoiesis and functionality of hematopoietic cells via autocrine or paracrine signaling[46,47]. Hematopoietic growth factors include colony stimulating factors (CSF), interleukins (IL), and cytokines. According to *in vitro* studies, different stages of hematopoiesis are supported by specific growth factors. Stem cell factor

(SCF) and FLT3 ligand are important for maintaining HSCs and early progenitors, which can differentiate into specific lineages upon the body's needs. On the contrary, the main growth factors for the lymphoid lineage are IL-7, IL-2, IL-5 and IL-15; whereas granulocyte-macrophage-CSF (GM-CSF) and IL-3 are the most important factors for the myeloid progenitor differentiation[48]. Further down the myeloid lineage, thrombopoietin and erythropoietin are known to stimulate production of platelets and erythrocytes from MEPs, while macrophage-CSF (M-CSF) and granulocyte-CSF (G-CSF) are the key factors for monocyte/macrophage[49,50] and neutrophil differentiation[51], respectively. The main growth factors and their functions are summarized in Table 1.

Table 1. *Function of the major hematopoietic growth factors (Adapted from Takei et al, Handbook of Hormones 2015)[52].*

Ligand	Receptor	Major target	Primary function
EPO	EPOR	Erythrocyte	Red blood cell production
G-CSF (CSF3, CSF-β)	CSF3R	Granulocytes, neutrophils	Neutrophil production
TPO (c-Mpl ligand)	TPOR	Megakaryocytes, platelets	Platelet production, HSC maintenance
GM-CSF (CSF2, CSF-α)	CSF2RA/B	Granulocytes, monocytes	Macrophage and granulocyte production
M-CSF (CSF1)	c-Fms (M-CSFRc, CSF1R)	Monocytes, macrophages, osteoclasts	Macrophage and osteoclast production
IL-5	IL-5R/IL5RA	Eosinophils, B cells, basophils	Eosinophil production
IL-3	IL3-R	Multilineage progenitors, basophils	Differentiation of immature myeloid progenitors

Hematopoietic growth factors regulate hematopoiesis by binding to their cognate receptors on target cells. Most growth factors signal through type I receptors of the cytokine receptor superfamily, which are recognized by four cysteine motifs of the extracellular domain[53,54]. Compared to other receptors, cytokine receptors form noncovalent connections with specific tyrosine phosphorylating enzymes called the Janus-associated kinases (JAKs). Upon ligand-induced receptor dimerization, JAK proteins (JAK1-3 and TYK2) induce a series of signal-transducing tyrosine phosphorylations[55]. Briefly, receptor associated JAKs are trans-phosphorylated followed by phosphorylation of the receptor's C-terminal tails. Subsequently, the phospho-tyrosines in the receptor form docking sites for Src-homology-2 (SH2) domain of signal transducer and activator of transcription (STAT) proteins. Upon binding to the receptor, STATs become phosphorylated

by JAKs, dimerize, and are released to the nucleus where they activate transcription of genes important for cell proliferation and survival[56,57]. Some of the most important genes induced by STATs are the *MYC* oncogene and the anti-apoptotic B-cell lymphoma-extra large (*BCL-XL*)[58,59]. Depending on the cellular context, different members of the JAK and STAT families (STAT1-4, STAT5A, STAT5B, STAT6) are activated by specific growth factors or by different stimuli.

STAT-mediated gene transcription is one of the key pathways for hematopoietic cell growth and development. However, JAKs also phosphorylate substrates of other major cell signaling pathways. The mitogen-activated protein (MAP) kinase pathway is a critical regulator of cell proliferation, whereas activation of the phosphatidylinositol 3 (PI3) kinase signaling leads to suppression of apoptosis[60].

The importance of JAK and STAT proteins on hematopoiesis has been demonstrated by genetic knockout studies on mice. Both *Jak1* and *Jak2* deficiencies cause lethal phenotypes[61]; mice with *Jak1* deficiency die perinatally and those with *Jak2* die as embryos. Furthermore, loss of *JAK3* is known to abrogate the development of T and B cells both in humans and mice[62]. Knockout of STAT genes also leads to failure in T cell differentiation and subsequent immunodeficiency phenotypes[63].

As the JAK/STAT pathway signaling has a central role in many biological processes, its functions are extremely well regulated. Three protein families, namely protein tyrosine phosphatases (PTPs)[64], protein inhibitors of activated STATs (PIAS)[65], and suppressor of cytokine signaling proteins (SOCS)[66] are known to control JAK-STAT signaling through dephosphorylation and competitive binding to the phosphotyrosine binding sites. Despite these control mechanisms, point mutations in these signaling molecules can lead to various hematological and immune disorders. The most commonly found aberrations in hematological malignancies are point mutations in *JAKs*, of which gain-of-function mutations of *JAK2* and *JAK3* are strongly associated with myeloproliferative disorders[67-69]. Importantly, *JAK2* V617F mutations are found in >80% of polycythemia vera cases, and 50-60% essential thrombocythemia and idiopathic myelofibrosis patients[69-71]. The clinical relevance of JAK mutations in myeloproliferative disorders has inspired the development of JAK-targeted therapies. This has led to the approval of JAK1/2 inhibitor ruxolitinib for the

treatment of myelofibrosis[72] besides other agents that are currently being tested in clinical trials.

2.1.1.3 Apoptosis

Hematopoiesis is a constant process that produces a vast amount of cells and therefore needs to be tightly controlled to maintain tissue homeostasis[73]. Apoptosis plays an important role for hematopoietic homeostasis by removing aged and damaged cells. Apoptosis can be initiated by two main pathways: through ligand binding to TNF-family death receptors (extrinsic pathway)[74] or via secretion of cytochrome c from mitochondria (intrinsic pathway). Both pathways activate caspases, protease enzymes that through a sequence of events lead to cell shrinkage, chromatin condensation and formation of apoptotic bodies. Ultimately, apoptotic bodies are removed by phagocytes[75]. In the extrinsic pathway, the cytoplasmic "death domain" of the receptor transmits death signals upon ligand binding[76]. The best characterized mediators of extrinsic pathway are FasL/FasR and TNF-α/TNFR1[75].

The intrinsic apoptotic pathway is triggered when suppression of death programs is lost, for example in nutrient deprived cells, or by stress stimuli caused by toxins, hypoxia and radiation. This leads to loss of mitochondrial membrane potential and subsequent release of pro-apoptotic proteins into the cytosol. Furthermore, pro-apoptotic proteins such as cytochrome c activate the mitochondrial caspase-dependent pathway[75]. Execution of apoptosis is controlled by members of the BCL-2 family that can alter mitochondrial membrane permeability. BCL-2 proteins can be divided into three groups based on their structural units called BCL-2 homology (BH) motifs. Anti-apoptotic proteins contain motifs BH1-4, pro-apoptotic effectors contain regions BH1-3, whereas the so called BH3-only proteins contain the BH3 motif, which is shared with the anti-apoptotic and pro-apoptotic proteins that they control[77] (Figure 2).

The first characterized anti-apoptotic protein was the oncogene BCL-2. It was discovered as part of the translocation *t*(14;18), associated with follicular lymphoma[78]. Later, other anti-apoptotic proteins (MCL-1, BCL-XL, BCLW, BFL1), pro-apoptotic proteins (BAX, BAK, BOK) and BH3-only proteins (BIM, BAD, BID, BIK, BMF, HRK, NOXA, PUMA) were discovered[79]. Importantly, overexpression of the anti-apoptotic proteins is one of the hallmarks of cancer that

provides growth advantage to cancer cells[80]. The BH3 motifs of anti-apoptotic proteins form a docking site for specific pro-apoptotic proteins. As cancer cells carry high levels of the apoptosis initiating pro-apoptotic proteins, they are referred to as 'primed for death'[81]. Triggering apoptosis in primed cells has hence been under intensive investigation for decades and has guided the development of BH3 mimetics[6]. These inhibitors replace pro-apoptotic proteins from cancer cells through competitive binding to the BH3 domain of anti-apoptotic proteins (Figure 2). The extensive work has led to the U.S. Food and Drug Administration (FDA) approval of a BCL-2 selective inhibitor venetoclax for treating relapsed/refractory chronic lymphocytic leukemia (CLL) patients, and more recently for elderly AML patients in combination with hypomethylating agents[82,83].

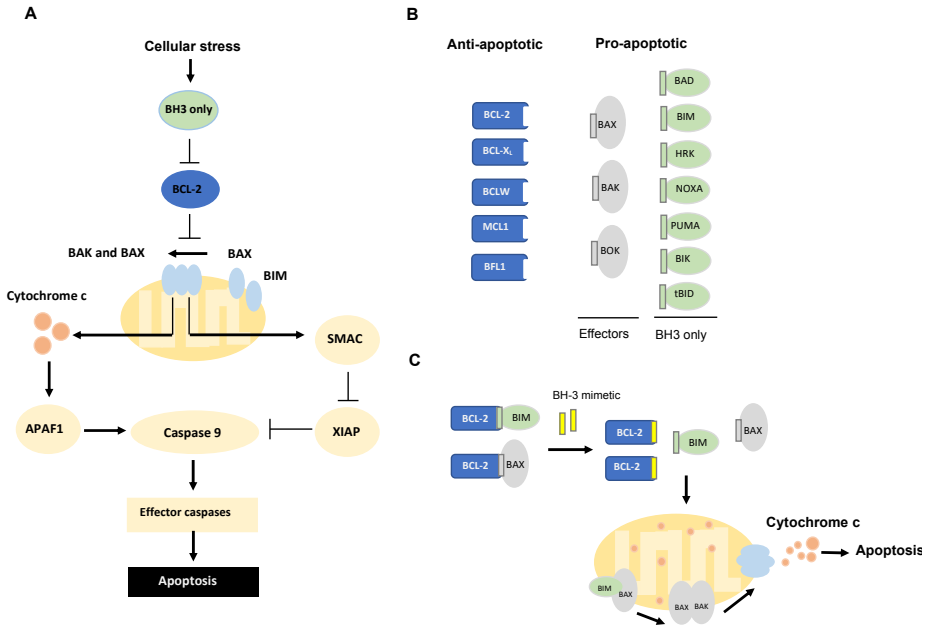

Figure 2. Intrinsic apoptotic pathway and mechanism of action of BH3 mimetics. (A) Cellular stress such as toxins and radiation activate BH3-only proteins, which inhibit anti-apoptotic BCL-2 proteins. This leads to activation and oligomerization of the effector proteins BAK and BAX at the mitochondria, driving mitochondrial outer membrane permeabilization (MOMP). MOMP allows the release of cytochrome c from the mitochondria into the cytosol, which through caspase activation results in apoptosis. In parallel, SMAC (second mitochondria-derived activator of caspase) represses inhibitor of apoptosis XIAP (X-linked inhibitor of apoptosis), which frees caspases and allows the activation of apoptosis. (B) Classification of the BCL-2 family proteins. (C) Mechanism

of action of BH3 mimetics such as venetoclax. Through binding to anti-apoptotic proteins such as BCL-2, the BH3 mimetics liberate pro-apoptotic proteins that can stimulate the activation of apoptosis. *Adapted from Ashkenazi et al, Nature Reviews 2017[6], Leverson et al, Cancer Discovery 2017[83] and Konopleva et al, Cancer Discovery 2016[84].*

2.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is hematological malignancy that develops from myeloid progenitors. Upon a malignant transformation, these cells lose their differentiation potential, and proliferate rapidly in the BM, eventually impairing normal hematopoiesis. Disruption of normal blood cell counts lead to life-threatening clinical symptoms such as anemia, thrombocytopenia and frequent infections[48,85]. AML is diagnosed when the amount of blast cells in the BM reaches >20% or specific cytogenic or molecular abnormalities are present (e.g. fusions *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA*). The diagnosis is confirmed by morphological characterization of the malignant cells with light microscopy and by flow cytometry. Cytogenetic and molecular genetic analyses are also performed routinely[48]. Out of all acute leukemias (myeloid and lymphoid) diagnosed in adults, AML is the most common type with ~ 20, 000 cases per year in the USA[86] and roughly 200 in Finland[87].

2.2.1. Origin and development

The evolution of AML can be occasionally traced back to HSCs that acquire somatic mutations as a result of aging and exposure to different environmental risk factors[88]. Clonal evolution studies using next generation sequencing (NGS) methods have detected many of these mutations to be random "passenger" mutations that lack a role in the pathogenesis of AML. In contrast, specific mutations that halt cell differentiation and increase clonal expansion have been termed as "founder mutations". These, so called type-II mutations occur in epigenetic regulator genes *DNMT3A, TET2, ASXL1*, *IDH1/2*[88-90] and splicing factor genes (*SF3B1*, *SRSF2*, *U2AF1*)[91]. In particular, epigenetic mutations are considered to be early founding events that may emerge even decades before the disease onset. Interestingly, 10% of healthy older individuals (> 65 years of age) harbor somatic mutations in the same epigenetic regulatory genes without dysplastic hematopoiesis[92,93]. This process of clonal hematopoiesis of

indeterminate potential has been associated with a modest risk of hematological malignancy and cardiovascular disease[93,94]. According to current understanding, the development of full-blown malignancy requires type-I "driver" mutations such as *FLT3*-ITD, which increase cell division. The necessity of complementing mutations is apparent from the fact that AML patients have on average three driver mutations at the time of diagnosis. Furthermore, as the disease evolves, the founder clone acquires cooperating mutations that give rise to subclones. As subclones carry different mutational burden, they contribute to the progression and relapse of the disease[88,89,95].

2.2.2 Role of the BM microenvironment in AML

The BM microenvironment is believed to influence the initiation and progression of myeloid malignancies. The majority of supporting evidence comes from mouse studies, which have characterized interactions between leukemic cells and stromal cells. Specifically, the role of osteoprogenitors in leukemogenesis is supported by gene knockout studies and studies exploring the effect of constitutive gene expression. Intriguingly, deletion of the endoribonuclease *Dicer1* gene from mouse osteoprogenitors was observed to induce the development of myelodysplastic syndrome (MDS) and in some cases AML in mice[96]. Loss of *Dicer1* led to reduced expression of ribosome maturation factor *Sbds*, which is known to be mutated in some patients with Shwachman-Bodian-Diamond syndrome. Interestingly, this syndrome is characterized by BM failure and predisposition to MDS and AML. Moreover, direct deletion of *Sbds* expression from osteoprogenitors induced myelodysplasia. In another study, activating mutation of β-catenin in mouse osteoblasts was seen to abrogate the differentiation potential of progenitor cells leading to development of AML[97].

Besides these findings from mouse studies, the role of stromal cells on leukemia induction in humans remain speculative. Indirectly, MSCs from MDS patients have been found to express lower levels of *DICER1, DROSHA* and *SBDS* compared to healthy controls[98]. In addition, 38.3% of MDS and AML patients were reported to accumulate β-catenin in osteoblast cells with enhanced Notch signaling[97] in support of the experimental models. One potential proof for the role of microenvironment in leukemogenesis comes from donor cell leukemia. In rare cases, allogeneic HSC transplantation (HSCT) may lead to development of

secondary leukemia[99,100], which seems to suggest that changes in the BM microenvironment may induce disease to healthy cells. However, the impact of germline mutations and drug-induced changes in the stroma have not been ruled out.

Leukemia cells interact with stroma and have been demonstrated to induce disease advancing changes in the microenvironment. Through secretion of angiogenic factors[101] and proinflammatory cytokines leukemia cells create an inflammatory environment that supports their survival and concomitant loss of normal HSCs. In a recent study, AML cells were shown to degrade the endosteal vasculature leading to loss of stromal cells and osteoblasts in the endosteal BM region[102]. Furthermore, AML cells in the vasculature poor region were speculated to be more resistant to chemotherapy[102]. Subsequent vessel rescue in mice was seen to improve efficacy of chemotherapy, pointing out the importance of BM niche alterations for leukemia growth.

2.2.3 Genomic landscape

The genetic landscape of AML is highly heterogenous with less than one recurrent gene-fusion event per patient (e.g. *PML-RARA*, *CBFB-MYH11* and *RUNX1-RUNX1T*)[103]. Large sequencing studies have attempted to identify recurrent mutated genes in AML to refine prognostic stratification of the disease and to identify previously unknown mutational events. Based on the first landmark study of 200 adult AML cases by The Cancer Genome Atlas (TCGA) Research Network, disease-defining mutations were found in 23 genes of nine different gene-categories[1]. Each patient carried on average 13 mutations of which only five were in frequently mutated genes. Interestingly, this showed that the number of mutations in AML genomes is much lower than in other cancers[2].

A few years after the TCGA study, Papaemmanuil and colleagues classified 1540 AML patients into 11 subtypes based on co-mutations[104]. This retrospective study led to the discovery of three new AML categories including chromosome-spliceosome mutations (18%), *TP53*-aneuploidy (13%) and conditional *IDH2*^{R172} mutations (1%)[104]. The genetic information from these NGS studies has deepened our understanding of AML genomics and has been incorporated into the World Health Organization (WHO) classification of AML. This scheme classifies AML into six main groups based on morphology and immunophenotypic, genetic and

clinical markers. The main groups of AML according to the updated WHO classification in 2016 are: 1) AML with recurrent genetic abnormalities, 2) AML with myelodysplasia-related changes, 3) therapy-related myeloid neoplasms, 4) AML not otherwise specified, 5) myeloid sarcoma, and 6) myeloid proliferations of Down's syndrome[105,106].

2.2.4 Therapy

Newly diagnosed AML patients are traditionally treated with cytarabine and anthracycline-based 7+3 induction chemotherapy (7 days of cytarabine plus 3 days of anthracycline). It remains the backbone treatment for most AML patients and leads to complete remission (CR) in 60-85% of younger patients and in 40-60% of older patients (>60 years of age)[85]. Treatment, however, may vary depending on patient-related factors, including advanced age[107], performance status, co-morbidities[107], and genetic factors. Genetic abnormalities strongly impact prognosis. Accordingly, the European LeukemiaNet (ELN) and the National Comprehensive Cancer Network (NCCN) have recently established risk groups for AML patients based on karyotype and mutational co-dependencies. Three risk groups are currently recognized, namely a) favorable, b) intermediate and c) adverse groups[108].

Recent approvals of targeted treatments have widened the therapy options for AML patients and certain patient groups are now eligible for an alternative first-line induction depending on their mutational and prognostic risk profile. Since 2017, favorable risk patients with core binding factor AML defined by rearrangements t(8;21) and inv(16)/t(16;16) that involve the *RUNX1/RUNX1T1* and *CBFB/MYH11* genes, are eligible for treatment with gemtuzumab ozogamicin (GO), a monoclonal antibody targeting CD33 conjugated to calicheamicin[109]. Treatment of intermediate risk patients is also changing with the approval of a multitargeted kinase inhibitor midostaurin with activity against *FLT3*-TKD and -ITD mutations. These mutations are found in approximately one third of all AML patients[110]. As *FLT3* mutations are among the most common genomic alterations in AML, development of targeted *FLT3* inhibitors has been a top priority in the post genomic era. After almost a decade of investigation, midostaurin in combination with chemotherapy was approved for the treatment of newly diagnosed *FLT3* mutated patients by the FDA[111,112]. Relapsed/refractory *FLT3*

mutation positive AML patients may be treated with a newer generation *FLT3*/AXL inhibitor giltertinib, which was also recently granted approval by the FDA[113]. Another modification to the 7+3-based induction therapy is that older AML patients with adverse-risk karyotype (therapy- or MDS-related AML, secondary AML) are now eligible for treatment with a liposomal form of daunorubicin and cytarabine at a 5:1 molar ratio. The liposomal drug CPX-351 has mainly been used as an induction treatment prior to allogeneic HSCT[114].

The B-cell lymphoma/leukemia 2 (BCL-2) inhibitor venetoclax was recently approved in combination with a hypomethylating agent or low dose cytarabine for treatment-naïve, unfit elderly AML patients. The approval was based on significantly improved overall survival in this high-risk patient group[115]. Venetoclax is a second generation BH3 mimetic that stimulates the intrinsic apoptotic pathway in leukemic cells through binding to BCL-2. This leads to subsequent release of pro-apoptotic proteins and induction of mitochondrial membrane permeabilization and apoptosis. The first generation BH3 mimetics (ABT-737 and ABT-263) were developed to target overexpressed anti-apoptotic BCL-2 family proteins BCL-XL, BCL-W and BCL-2 in hematological malignancies. The safety of these agents, however, was limited due to inhibition of BCL-XL in platelets and subsequent thrombocytopenia[116]. Intriguingly, venetoclax targets only BCL-2 and has been shown to have an acceptable safety profile in AML. While single agent venetoclax treatment of relapsed/refractory AML yielded only a modest response rate of 19% and a median complete remission of 48 days[84], approximately 70% of treatment naïve, elderly AML patients were shown to benefit from venetoclax in combination with hypomethylating agent[115,117]. This drug combination also had a favorable safety profile, with febrile neutropenia being the most frequent adverse side effect. Based on these results, venetoclax can now be used for the treatment of elderly, newly diagnosed AML patients, in addition to relapsed/refractory CLL patients with 17p deletion.

Since the discovery of recurrent mutations in *IDH1* and *IDH2* in approximately 20% of AML patients[1,104,118,119], targeted therapies have been actively developed against these aberrations, which contribute to overproduction of the oncometabolite 2-hydroxyglutarate. The development work has led to the approval of *IDH2* inhibitor enasidenib in 2017 by the FDA[120], followed by the approval of *IDH1* inhibitor ivosidenib in 2018[121]. Both therapies have been approved for the treatment of relapsed/refractory AML patients and have resulted

in CR or CR with incomplete hematologic recovery (CRi) in 20% of patients treated with enasidenib and 30% of patients receiving ivosidenib. In May 2019, ivosidenib was approved as the first-line treatment for *IDH1* mutated AML. Currently, both therapies are being explored further in clinical trials as single agents and in combination with other therapies, with the goal to achieve CR prior to allo-HSCT.

2.3 Therapy resistance in AML

Despite the recent improvements in the treatment of AML, the disease reappears in approximately 50% of younger patients (≤ 60 years) and 80-90% of older patients due to development of drug resistance[122,123]. In older patients, the long-term survival with standard chemotherapy remains poor with only 10-15% reaching permanent cure[108]. Although HSCT can potentially prevent disease recurrence, only one-fourth of patients are eligible for intensive salvation therapy and transplantation[124]. Implementation of targeted therapies for the treatment of AML has also been challenging due to disease heterogeneity. As the disease is burdened by various co-occuring mutations, targeting one mutation or pathway has not resulted in major impact on the outcome. Responses to many of the treatments have remained short-lasting due to emergence of resistance. For these reasons, most relapsed AML patients ultimately die from the malignancy and there is substantial need for new treatment strategies to overcome therapy resistance.

2.3.1 Mechanisms of drug resistance

Drug resistance remains a major challenge for the treatment of AML. Resistance can arise either intrinsically prior to therapy due to pre-existing resistance-mediating mutations or can be acquired as a consequence of therapy. Chemotherapy can enhance the selection and enrichment of the resistance bearing clones that lead to disease progression[125]. Other mechanisms that induce drug resistance in cancer cells include increased repair of DNA damage, epigenetic changes, resistance to apoptosis, modifications to drug targets and drug metabolism (intake, efflux and detoxification)[126].

In AML, mutations to enzymes that phosphorylate cytarabine to its active form (cytarabine triphosphate) can lead to inactivation of the drug and subsequent cytarabine resistance[127]. Importantly, chemotherapy can induce development of cross-resistance to a range of drugs with different mechanisms of action. Multidrug resistance is mainly caused by overexpression of adenosine triphosphate (ATP) binding cassette (ABC) membrane transporters that increase cellular drug efflux[128]. Acquisition of mutations is another way for cancer cells to escape drug toxicity. One of the best-known examples of this mechanism is imatinib resistance in chronic myeloid leukemia (CML) with *BCR-ABL*. Point mutations and amino acid substitutions to the BCR-ABL kinase domain lead to structural changes in the protein, which compromise binding of imatinib to its target[129]. Similar to imatinib, mutations to the ATP-binding site and activating loop residues of *FLT3* kinase domain lead to FLT3 inhibitor resistance in AML.

Continuous exposure to drugs can also contribute to the development of acquired drug resistance by inducing mutations to the BCL-2 family genes. More specifically, these mutations may upregulate expression of anti-apoptotic proteins or reduce expression of pro-apoptotic proteins leading to BCL-2 inhibitor resistance. In cell line studies, resistance against BCL-2 inhibitor venetoclax has been associated with overexpression of anti-apoptotic BCL-XL and MCL-1. In addition, mutations to pro-apoptotic proteins, in particular the BH3 binding domain, have been shown to hinder venetoclax binding to its target[130,131].

Besides intracellular drug resistance mechanisms, extrinsic factors of tumor microenvironment may interact with leukemic cells and protect them from drug induced cell death. This phenomenon is showcased by the fact that AML cells are retained in the tumor microenvironment via soluble factor- or cell adhesion-mediated interactions since blast cells express many of the same adhesion molecules as normal HSCs (e.g. CXCR4, VLA-4, CD44)[5,132]. A well-known example of cell adhesion mediated resistance mechanisms is the CXCL12/CXCR4 pathway. The impact of this pathway on chemoresistance is shown with co-culture models, which have demonstrated upregulated expression of CXCR4 in AML cells after chemotherapy[133,134]. In this model, interaction of CXCR4 with CXCL12 expressing stromal cells and retention to the protecting BM was abrogated by CXCR4 inhibitors, which sensitized cells to chemotherapy[133,134]. The same was shown in murine models[135]. Moreover, increased CXCL12/CXCR4 and basic fibroblast like growth factor (FGF2) signaling has been shown to contribute to *FLT3* inhibitor resistance in *FLT3*-ITD

patient samples in addition to other survival pathways[136-138]. Examples of interactions of leukemic cells with the stroma that enhance chemotherapy resistance include VLA-4 and CD44 adhesion factor mediated binding of leukemic cells to the extracellular matrix component fibronectin[139,140], and VLA-4/VCAM-1 interaction with the stromal cells[141]. Likewise, VLA-4 negative patients are known to have favorable prognosis as they do not develop BM minimal residual disease induced by the leukemia-stroma interaction[139]. Interactions between leukemic cells and the stroma are summarized in Figure 3.

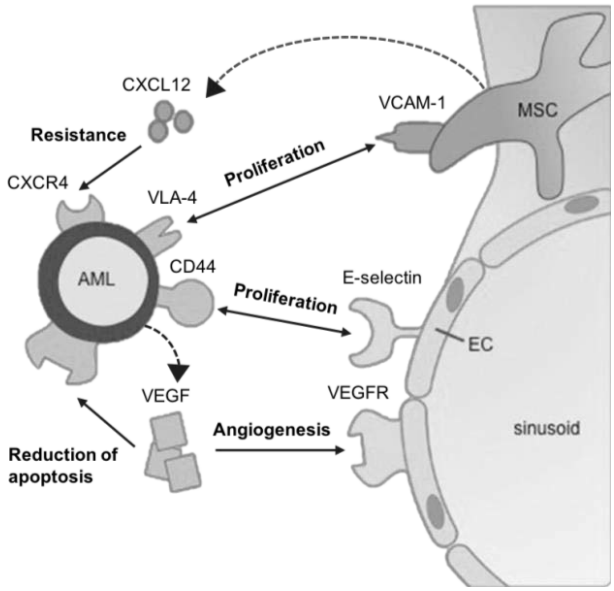

Figure 3. Interactions of AML cells with the surrounding BM microenvironment. AML cells are retained in the tumor microenvironment via soluble factor- and cell adhesion-mediated interactions. Endothelial cell, EC; mesenchymal stromal cell, MSC; vascular endothelial growth factor, VEGF; vascular endothelial growth factor receptor, VEGFR; C-X-C motif chemokine 12, CXCL12; C-X-C chemokine receptor 4, CXCR4; very late antigen 4, VLA-4; vascular cell adhesion molecule 1, VCAM-1. *Adapted from Behrmann et al, Frontiers in Oncology 2018*[142].

The BM microenvironment is enriched with many soluble factors including cytokines and chemokines. The role on these factors on drug resistance has been studied with cell line models. Interestingly, one study has reported that G-CSF may protect AML cells from c-KIT/ABL inhibitor (imatinib and nilotinib) mediated cell death[143]. Similarly, stroma-derived factors were shown to cause

imatinib-resistance by stimulating STAT3 phosphorylation and expression of anti-apoptotic proteins in CML cells. Furthermore, inhibition of paralleling JAK/STAT signaling by JAK1/2 inhibitor ruxolitinib was shown to restore CML cell sensitivity to tyrosine kinase inhibition[144]. Another recent study observed GM-CSF and IL-3 signaling through JAK, STAT5 and PIM kinases to rescue *FLT3*-ITD AML cells from FLT3 inhibition[145]. These and other clues indicate that the BM microenvironment blocks FLT3 inhibition through activation of redundant signaling pathways such as the RAS/RAF/MEK/ERK[146] and PI3K/AKT/mTOR[147,148] that lead to survival of *FLT3*-ITD AML cells and eventual relapse.

2.3.2 Targeted therapies in clinical development

Many novel compounds, which have been designed to target chemoresistant leukemic stem cells are currently being tested in clinical trials (Figure 4). Epigenetic compounds under development include histone deacetylation (HDAC) inhibitors, such as pracinostat, which is evaluated in combination with hypomethylating agents for older, unfit patients[149]. The novel hypomethylating agent guadecitabine has shown promising results in elderly AML patients and relapsed/refractory AML due to its improved resistance against cytidine deaminase degradation[150,151]. Bromodomain and extraterminal (BET) protein inhibitors that control RNA polymerase II and transcription of oncogenes are also under evaluation[152]. However, as a single agent, treatment with the BET inhibitor OTX-015 (birabresib) was reported to yield CR/CRi in only 3 out of 36 AML patients[153]. Potential combination therapies and possible predictive biomarkers indicating OTX-015 response need to be further investigated.

As the majority of AML cases show constitutively activated receptor tyrosine kinase signaling, development of small molecule inhibitors against kinase activities has been of key interest during the past two decades. Most of the work has been directed towards FLT3 and KIT inhibitors, as alterations to these genes are frequent in AML. This has led to the development of new generation FLT3 inhibitors with improved specificity against FLT3 compared to the first-generation agents. Currently, three next-generation FLT3 inhibitors (crenolanib, quizartinib, giltertinib) are in phase III clinical trials for the treatment of AML[154]. Dasatinib, a multikinase inhibitor with activity against the KIT receptor tyrosine kinase (CD117) is also in a phase III clinical trial (NCT02013648) investigating

chemotherapy for the treatment of core-binding factor (CBF) AML[155]. KIT is highly expressed and commonly mutated in CBF-AML[156], providing the rationale to add dasatinib to the current induction and consolidation chemotherapy regimen.

Recently, immune-based therapies have been under careful scrutiny for AML treatment in hope of harnessing the body's own immune system against leukemia. Current immunotherapies in clinical trials include 1) monoclonal antibodies targeting blast specific antigens and 2) T-cell response enhancing therapies such as bispecific T-cell engagers, immune checkpoint receptor antibodies and chimeric antigen receptor (CAR) T-cells[157]. Monoclonal antibodies have been developed against CD33 (e.g. lintuzumab) and CD38 (e.g. daratumumab). Other antibody-based approaches use antibodies to deliver toxic chemicals into leukemic blasts such as the CD33 drug conjugate gemtuzumab ozogamicin (GO) that is the only currently approved immunotherapy for AML[158,159]. Therapies in current clinical development include bispecific antibodies recognizing blast specific CD33[160,161] and CD123[162] antigens, conjugated with T-cell antigen CD3, in addition to antibodies blocking immune checkpoint receptors, namely the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1) on T-cells[163,164].

Lastly, therapeutic strategies are being developed to reverse the protective role of the BM microenvironment and its function as a sanctuary for residual disease and tumor cell survival. Most progress has been achieved so far with CXCR4 inhibitors, as high CXCR4 expression is a prognostic indicator for poor outcome in AML[165,166]. Of these inhibitors, plerixafor has been approved for HSC mobilization in conjunction with G-CSF for lymphoma and myeloma[167,168] and has been tested for AML patients in the first-line and salvage setting[169-171]. Despite their acceptable safety profile, larger randomized trials are needed for determining the benefit of CXCR4 inhibitors and for finding optimal combination regimens. Another significant advance in targeting the BM microenvironment has been made with the development of inhibitors against the adhesion molecule E-selectin. GMI-1271 is an E-selectin antagonist that disrupts blast cell binding to BM endothelial cells and subsequent activation of cell survival pathways[172,173]. Phase I/II clinical trials on relapsed/refractory and newly diagnosed, older AML patients obtained CR/CRi of 47% in combination with chemotherapy[174,175]. This led to a breakthrough therapy and orphan drug designation by the FDA and European Commission in 2017, and is currently followed up in an ongoing phase III clinical

trial to evaluate the efficacy of GMI-1271 plus chemotherapy in relapsed/refractory AML patients (NCT03616470).

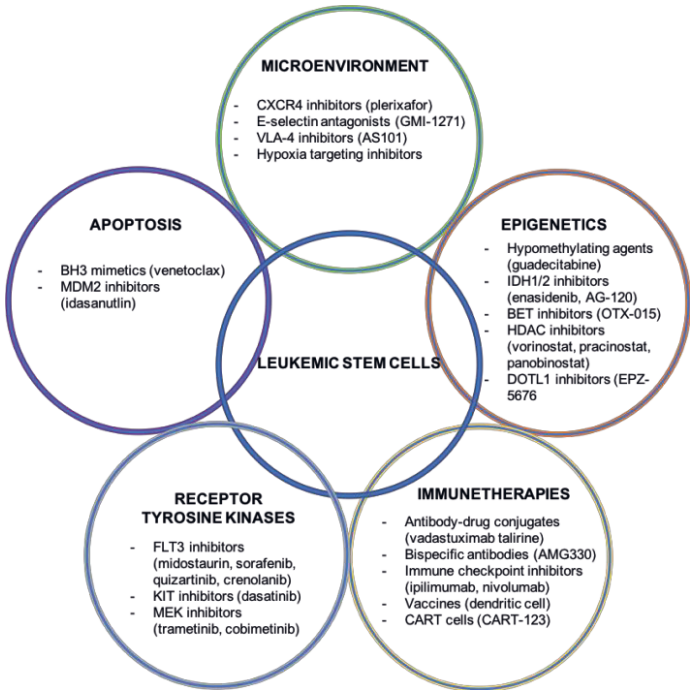

Figure 4. Novel therapy areas developed for the treatment of AML. Targeting therapy resistant leukemic stem cells is crucial for obtaining long term cure. *Adapted from Short et al, Lancet 2018*[3].

2.4 Drug sensitivity testing in hematological malignancies

Various drug screening methods have been developed to reliably assess the potency, selectivity, and efficacy of anti-cancer drugs *ex vivo*. Much of the developmental work has been done with leukemic cells due to the feasibility of harvesting and culturing cells collected from blood stream compared to solid tissues. The efficacy of anticancer drugs on leukemia cells can be assessed with assays that measure cell viability[176]. The most common assays are based on measurement of cell mass with the protein dye sulforhodamine B, detection of metabolically active cells through formation of a purple salt (MTT assay and

related assay formats), or release of ATP[177]. Out of these assays, ATP quantification most accurately quantifies the number of live cells. In addition to cell viability-based assays, also cell proliferation, apoptosis, and differentiation can be measured by flow cytometry (FACS), using different fluorochromes or fluorescent marker proteins. Flow cytometry-based assays benefit from the ability to monitor specific drug responses of particular cell populations, in comparison to assays measuring total cell killing. Recently, methods using high-throughput immunofluorescence microscopy for drug sensitivity testing have also emerged as a promising way to guide treatment selection for leukemia patients, although these methods warrant further investigation in larger patient cohorts[178].

The optimal readout and response parameters of different drug sensitivity testing assays remain to be standardized. Typically, half-maximal inhibitory or effective concentration (IC50/EC50) or area under the curve (AUC) measures have been used as a parameter to analyze drug response. These parameters, however, inaccurately represent differences in drug response patterns by for example neglecting maximal inhibition. Moreover, *ex vivo* culture conditions of primary cells require optimization as leukemic cells tend to differentiate, become senescent, or enter spontaneous apoptosis that can modify drug responses *ex vivo*. For example, cell culture oxygen concentration may impact cell metabolism and the production of reactive oxygen species altering the function of cells[179]. It is also important to consider the cytokine content and interactions with the BM microenvironment in high content drug testing assays as cells *in vivo* are in contact with many cell types. Improved co-culture methods that address the role of the microenvironment and immune system on drug responses are hence needed for better evaluation of the therapeutic outcome of drug treatment on patients.

In recent years, high throughput drug screening platforms have been utilized for finding molecularly targeted treatments for leukemia patients. By combining measurements of drug sensitivity responses with molecular profiling, results from such testing have facilitated repurposing of approved drugs to other disease entities[180]. For example, *ex vivo* drug sensitivity testing led to identification of the tyrosine kinase inhibitor axitinib as a successful treatment option for CML patients with T315I mutation in the *BCR-ABL1* fusion gene[181]. Regardless of the few success stories, more innovative *ex vivo* drug testing methods are needed, which better reflect the *in vivo* microenvironment, in order to more successfully translate results from *ex vivo* drug screening into patient care.

3 AIMS OF THE STUDY

The overall aim of this thesis was to study the impact of the BM microenvironment on AML cells' drug responses and to investigate mechanisms and predictors of drug resistance in addition to novel biomarkers for AML with the following specific aims:

1. Evaluation of the effect of stroma-derived factors on *ex vivo* drug responses of primary AML cells.

2. Determination of molecular mechanism and biomarkers of resistance to BCL-2 inhibitors.

3. Identification of combination therapies to overcome *ex vivo* BCL-2 inhibitor resistance of AML patient samples.

4. Discovery of protein biomarkers linked to AML.

4 MATERIALS AND METHODS

4.1 Study specimens

4.1.1 Patients

BM aspirates and peripheral blood (PB) samples used for the studies were obtained from AML patients after receiving their informed consent. BM aspirates from healthy donors were used as controls. Procedures were performed according to approved protocols (institutional review board of Helsinki University Hospital and Comprehensive Cancer Center) and in accordance with the Declaration of Helsinki.

Study I included BM aspirates and PB samples (n = 26) from 21 AML patients. Drug sensitivity was assessed for 18 samples from 13 AML patients. Four of the samples were collected at the time of diagnosis whereas rest of the samples were from relapsed or refractory patients (10 relapse, 3 refractory). Other samples, not included in the drug sensitivity study, were used for mechanistic studies. In study II, RNA sequence derived gene expression profiles were compared with the drug sensitivity profile of 32 AML patient samples in order to identify associations with venetoclax response. Furthermore, mRNA-based gene expression analysis was done for samples from 112 AML patients and 4 healthy individuals for studying expression of the *S100* family genes. In study III, datasets from the study of Foss *et al*[182], including protein and transcriptomic data of four AML patients and six healthy donors, were reanalyzed for finding links to hematopoietic malignancies. In addition, 12 AML patient samples and one healthy control sample were used for validation experiments.

4.1.2 Cell lines

HS-5 human stromal cell line (American type culture collection) was used in study I for producing conditioned medium (CM). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and

penicillin-streptomycin until 70-80% confluent, following media replacement and collection after 72 h. Study II used leukemia cell lines MOLM-13, Kasumi-1, SKM-1, NOMO-1, SHI-1 and ML-2 (Deutsche Sammlung von Mikroorganismen und Zellkulturen Braunschweig, Germany). The cell lines were maintained in their respective media; SHI-1 cell line in IMDM medium with 20% FBS, the other cell lines in complete RPMI 1640 medium with 10-20% FBS. All the cell lines were maintained at 37 °C and 5% CO_2.

4.1.3 Animals

The animal experiments in study I were approved by the Norwegian State Commission for Laboratory Animals. In order to study drug resistance to BCL-2 inhibitors, female non-obese diabetic/severe combined immunodeficient γIL2rγ^{null} (NSG) mice were inoculated IV with MOLM-13^{luc} AML cells and used as a murine AML model in the study. One week after tumor implantation, the mice were treated with 25 mg/kg venetoclax (intraperitoneally; vendor ChemieTek), 50 mg/kg ruxolitinib (by mouth; vendor ChemieTek), venetoclax/ruxolitinib combination or a vehicle (n = 6 per group) for 3 weeks, 5 days a week. Tumor growth was monitored weekly by whole-body imaging (IVIS Spectrum Imager, PerkinElmer), detecting luciferin signal of the tumor cells.

4.2 Sample processing

Mononuclear cells (MNC) were isolated from BM and PB samples by density gradient separation (Ficoll-Paque PREMIUM, GE Healthcare). Cells were maintained in mononuclear cell medium (MCM, PromoCell), or in a mix of 25% HS-5 and 75% RPMI 1640 medium (study I). Excess cells were vitally frozen in FBS/10% DMSO and stored in liquid nitrogen until used for experiments.

4.3 Drug sensitivity and resistance testing (DSRT)

Drug sensitivity and resistance testing was done according to previously published method by Pemovska *et al*[180] Briefly, drugs were pre-dispensed onto 384-well cell

culture plates in five different concentrations covering a 10,000-fold concentration range with an acoustic liquid handling device (Echo 550, Labcyte Inc.). AML cells were seeded to the drug plates with a Multidrop Combi peristaltic dispenser (Thermo Scientific). In study I, the drug testing was done in both MCM medium and 25% HS-5 conditioned medium/RPMI medium (CM), at a cell density of 4 x 10^5 cells/mL. After 72 h incubation at 37 °C, 5% CO_2, cell viability was measured using the CellTiter Glo assay (Promega) that leads to cell lysis and release of ATP from viable cells. Luminescent signal proportional to the amount of ATP and metabolically active cells was then quantified with a PHERAstar plate reader (BMG Labtech). Dose response curves were generated based on the cell viability readouts with the Dotmatics software (Dotmatics Ltd.)

4.3.1 Compound library

A compound collection consisting of 304 FDA/EMA approved and investigational oncology drugs was used in the studies I and II. The compounds were obtained from the National Cancer Institute Drug Testing Program or purchased from chemical vendors. Based on instructions, the drugs were dissolved in DMSO or water and stored in a desiccator protecting them from humidity.

4.3.2 DSRT data analysis

Drug sensitivities were quantified with a drug sensitivity score (DSS), which corresponds to the modified calculation of the area under the curve, described previously by Yadav *et al*[183]. The DSS considers all four curve fitting parameters (IC50, slope, minimum and maximum response) in the calculation of AUC, in relation to the area between the 10% threshold and 100% inhibition. DSS values range between 0 and 50; 0 indicating less than 10% response and 50 the maximum inhibitory response. Leukemia specific drug responses were obtained by comparing DSS values of patient samples to mean DSS of healthy donor samples (selective DSS, sDSS). In study I, Ward's algorithm and Spearman (drugs) and Manhattan (patients) distance measures were used for clustering the patient samples based on drug sensitivity differences between CM and MCM (deltaDSS). Comparison of DSS values with or without CM across samples was performed

using paired Student's *t* tests. *P*-values <0.05 were considered statistically significant.

4.3.3 Drug combination testing

In studies I and II, drug combinations were tested by adding two drugs simultaneously at fixed concentrations to AML cells. Subsequently, cell viability data were analyzed with the Zero Interaction Potency (ZIP) model described by Yadav *et al*[184]. Synergistic drug combinations were determined by the δ synergy score, indicating the difference between the observed and expected response, and visualized by pseudo-coloring a 2-dimensional contour plot over the dose matrix.

4.3.4 Co-culture assays

MSCs from AML patients were seeded onto 96-well plates (Corning, Corning, NY) at a density of 1.5 x 10^5 cells/mL and let to adhere overnight, after which cells from AML patient were added directly to the stroma (1 x 10^5 cells/100 mL/well) or separated by a 0.4-mm pore membrane (Corning). Drugs (0.1% dimethyl sulfoxide, 100 μM benzethonium chloride, 300 nM ruxolitinib, 100 nM venetoclax) were added to the co-cultures and incubated with the cells for 48 hours. AML cells were labeled with blast cell markers PE-Cy7-CD34 and BV605-CD45 and viable cells were distinguished from apoptotic and dead cells with PE-Annexin V and 7AAD antibodies (all antibodies from BD Biosciences). Flow cytometric analysis was done using the iQUE Plus instrument (Intellicyt, Albuquerque, NM) and ForeCyte software (Intellicyt).

4.3.5 Prediction of genetic determinants of drug response

In study II, a linear regression model[185] was applied to identify genes associated with drug response (or failure to respond). To find the true relationship between gene expression change and drug sensitivity, we corrected confounding factors including age, gender, sequencing batch, RNA extraction method and RNA-sequencing library preparation in the linear regression model. Differentially expressed genes with a false discovery rate (FDR) < 0.05 were considered

significant for drug sensitivity association. Unsupervised hierarchical clustering of samples and genes was done using the Ward's method with Euclidean distance measure.

4.4 Gene expression analysis

RNA was extracted from AML cells using the miRNeasy kit (Qiagen; study I) or the NucleoSpin RNA kit (Macherey-Nagel; study II) and quantified using the Qubit RNA BR Assay Kit (Thermo Fisher). RNA integrity was assessed with the RNA 6000 Nano Kit and Bioanalyzer (Agilent).

4.4.1 RNA sequencing

RNA was depleted of ribosomal-RNA (Ribo-Zero™ rRNA Removal Kit, Epicentre), purified (RNeasy Clean-up Kit, Qiagen) and reverse transcribed to double stranded cDNA (SuperScript™ Double-Stranded cDNA Synthesis Kit, Thermo Fisher). RNA sequencing libraries were prepared using Illumina compatible Nextera™ Technology (Epicentre), size-selected and purified (QIAquick Gel Extraction kit, Qiagen). Transcriptomes were sequenced with the Illumina HiSeq2000 platform, using the TruSeq SBS Kit v3-HS reagent kit for paired end sequencing with 100 bp read length.

RNA sequencing data were processed as described by Kumar *et al*[186]. Briefly, reads were corrected with Trimmomatic[187] and after filtering, aligned to the human genome (GRCh38) using STAR[188] and EnsEMBL v82 gene models. Picard was used for sorting the aligned reads and marking PCR duplicates. Feature reads were computed with SubRead[189] and transformed to expression estimates with Trimmed Mean of M-values (TMM) normalization[190]. Genomic features with counts per million (CPM) value $\leq$1.00 were excluded.

4.4.2 Real-time quantitative PCR analysis

Complementary DNA was prepared from total RNA using SuperScript III

Reverse Transcriptase (Thermo Fisher). Real-time quantitative PCR (RT-qPCR) was performed on 10 ng of cDNA using iQ SYBR Green Super Mix (Bio-Rad, Hercules, CA, USA) in a CFX 96 instrument (Bio-Rad). The most stable reference genes were determined by CFX manager software from Bio-Rad and used for normalization purposes. Standard curves for all RT-qPCR reactions were included to determine specific primer efficiencies.

4.5 Protein analysis

4.5.1 Cytokine analysis

RayBio C-Series Human Cytokine Antibody Arrays C2000 and C5 (RayBiotech) were used for analyzing the cytokine content of different media and BM supernatant fluids in study I. Signal was detected by using chemiluminescence and quantified with the Odyssey imaging system (LI-COR Biosciences).

4.5.2 Western blotting

MNCs were lysed in RIPA buffer (Cell Signaling Technology) containing additional 1 mM phenylmethylsulfonyl fluoride (PMSF). Following sonication and addition of Laemmli buffer, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% gel, Bio-Rad), and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% milk (studies I and II) or 5% bovine serum albumin (study III) for 1 h. Detection antibodies used included: study I, anti-MCL1 (#4572), anti-BCLXL (#2764) (Cell Signaling Technology), anti-BCL2 (Dako), anti-β-actin (C4, sc-47778; Santa Cruz Biotechnology); study II, anti-calgranulin A (C-10, sc-48352), anti-calgranulin B (MRP 1H9, sc-53187) (Santa Cruz Biotechnology), anti-β-actin (Merck); study III, DDX6/RCK, GPX1, fumarase (Cell Signaling Technology) and anti-GAPDH (Santa Cruz Biotechnology). Proteins were visualized with the Odyssey imaging system (LI-COR Biosciences).

4.5.3 Phospho-flow analysis

After 20 minutes of cytokine stimulation (10 ng/mL; Peprotech), AML patient cells were fixed with Lyse/Fix Buffer (BD Biosciences) for 10 minutes and permeabilized with Perm Buffer III (BD Biosciences) for 30 minutes at -20°C. Cells were washed, stained with Alexa 647-anti-phospho-Stat5 (pY694), PE-CF594-anti-phospho-Stat3 (pY705), BV421-anti-phospho-Akt (pS473), and PE-anti-phospho-Erk1/2 (pT202/pY204; all antibodies from BD Biosciences), and analyzed on the iQUE Plus instrument (Intellicyt). Data were analyzed with Cytobank (Fluidigm).

4.6 Pathway and network analysis

In study II, Enrichr analysis tool and Ingenuity Pathway Analysis were used for gene function and network analysis of 349 genes negatively associated with venetoclax response. Enrichment analysis was done using Kyoto Encyclopedia of Genes and Genomes (KEGG) 2016 and Reactome 2016 gene set libraries. Similarly, Ingenuity Pathway Analysis was used in study III to visualize and predict downstream effects of candidate biomarkers from proteomic and transcriptomic datasets.

4.7 Statistical analysis

GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) was used for statistical analysis in study I, whereas in study II, the analyses were performed with R version 3.3.3 (2017-03-06) and Prism 7. Statistical dependence between two variables was assessed by Pearson's correlation coefficient modeling. The Mann Whitney *U* test was used for analyzing differences between drug responses and gene expression. In study II, the Wilcoxon signed-rank test was applied to find significant difference in drug response between wild type and mutated AML patient samples for a given mutation. In study III, ROTS was used as the statistical data analysis procedure for analyzing common protein-transcript pairs from the study of Foss *et al*[182]. *P*-values below 0.05 and FDR below 0.05 were considered statistically significant.

5 RESULTS

5.1 BM stroma-induced protection of AML cells (I)

The BM microenvironment plays a role in supporting the survival of leukemic cells and influencing their responses to therapeutic agents. In study I, we aimed to develop a BM stroma-derived culture condition for high-throughput drug testing to evaluate the effect of BM microenvironment on drug responses in AML.

5.1.1 Development of *ex vivo* culture conditions

Ex vivo cell culturing conditions impact the survival and responsiveness of leukemic cells to therapeutic agents. Outside the body, leukemic cells are more prone to spontaneous, culture-induced apoptosis in the absence of growth factors and interactions of the BM microenvironment. Although leukemia cells can be supplemented with individual myeloid growth factors or combinations of them, their ability to support cell survival and feasibility in *ex vivo* experiments is limited[191,192]. Direct culturing with human stromal cell lines has been shown to improve AML cell viability[193], however implementation of co-culture models to high-throughput testing with hundreds of drugs is challenging. Hence, in study I, we aimed to improve *ex vivo* culturing conditions of AML patient cells for high-throughput DSRT and evaluated the impact of conditioned medium (CM) from a human BM stroma cell line HS-5 on AML cell viability.

CM from the stromal cell line HS-5 was previously shown to support the proliferation of hematopoietic progenitor cells.[191] In our study, we observed that 25% CM combined with RPMI 1640 medium could significantly increase AML cell viability over three days culturing time compared to commercially available MCM. Furthermore, vitally frozen AML patient samples had an improved recovery in 25% CM after thawing, facilitating the use of frozen AML patient samples for *ex vivo* experiments (Figure 5A-B).

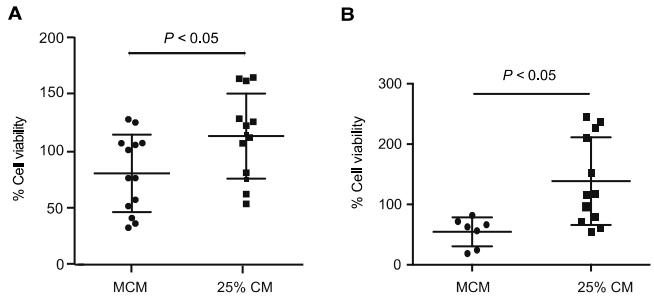

Figure 5. HS-5 CM supports survival of primary AML patient samples. (A) Freshly isolated or (B) vitally frozen AML cells were cultured in MCM or 25% HS-5 CM/75% RPMI mix for 3 days, and cell viability was assessed with the CTG assay.

5.1.2 Stromal cell secreted cytokines stimulate STAT5 phosphorylation and JAK/STAT signaling

In order to identify the key growth factors in HS-5 CM that support AML cell survival, we analyzed the cytokine content of the CM with an antibody panel detecting 174 human cytokines. The stromal cell CM was detected to contain high levels of interleukin-8 (IL-8), GRO, IL-6, GM-CSF, MCP-1, MIP-3α and G-CSF (Figure 6A). To follow up on the physiologic relevance of the detections, we analyzed the cytokine content of a BM supernatant fluid from a healthy donor and a patient with AML, in addition to CM collected from patient derived MSCs. Most of the abundant cytokines in HS-5 CM were detected in BM fluid from AML patients and CM from MSCs, although at lower level compared to the HS-5 CM. Moreover, expression of corresponding receptors for the abundant cytokines indicated that these cytokines are likely to affect proliferation of AML cells. To further investigate these findings, we assessed the impact of stroma-derived soluble factors on cellular signaling activation. AML cells were stimulated with 25% CM or with individual cytokines and the phosphorylation of STAT3, STAT5, ERK and AKT was measured in order to evaluate the activation of JAK/STAT, RAS/RAF/MEK/ERK and PI3K/AKT/mTOR cell signaling pathways, respectively. Compared to control conditions, CM induced rapid phosphorylation of STAT5. This effect was mimicked by the addition of GM-CSF or G-CSF to

cell culture medium, demonstrating the importance of JAK/STAT signaling pathway on leukemic cell survival (Figure 6B-C).

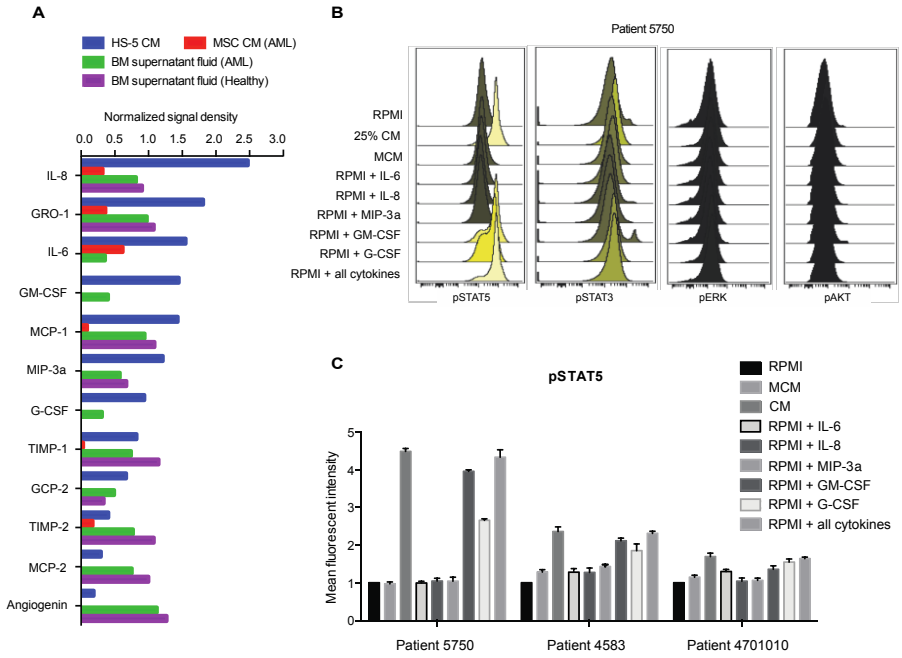

Figure 6. BM stromal cell–CM increases JAK/STAT signaling in primary AML patient samples. (A) Cytokine levels measured from HS-5 CM, CM from primary MSCs of an AML patient, and BM supernatant fluid collected from one AML patient and a healthy donor. Levels normalized to detections from RPMI 1640 medium. (B) Phospho-flow analysis of phospho-ERK, phospho-AKT, phospho-STAT3, and phospho-STAT5 in AML patient cells treated with RPMI, 25% HS-5 CM, MCM, or RPMI supplemented with 10 ng/mL of the indicated cytokines or a combination of all the cytokines for 20 minutes, showing stimulation of STAT5 phosphorylation. (C) Detection of STAT5 phosphorylation in AML cells from three patients after 20 minutes of stimulation with different media conditions or individual cytokines (10 ng/mL). Error bars represent standard deviation of duplicates.

5.1.3 Impact of stroma-derived factors on *ex vivo* drug responses of AML cells

In order to evaluate the effect of BM soluble factors on response to different drug classes, we tested a collection of 304 inhibitors including 136 drugs (45%) approved by FDA/EMA, 103 (34%) investigational agents and 65 probes (21%) for 18 AML patient samples in the 25% CM condition. Drug responses were compared to results obtained from parallel testing in a standard MCM medium to extract the stroma specific effects on drug sensitivity. Drug response differences were evaluated by drug sensitivity scores (DSS), which were calculated from dose-response parameters.[183] Overall, response to 12% (36/304) of the drugs was significantly affected (FDR < 0.05) by the drug screening condition as quantified by the difference of mean DSS (deltaDSS) for each drug in both conditions (Table 2).

Table 2. *Analysis of the differences in AML patient samples' sensitivity to drugs in HS-5 CM or MCM medium.*

Drug	Mechanism/ targets	Included pairs	Mean DSS CM	Mean DSS MCM	Mean DSS difference	FDR
Ruxolitinib	JAK1/2	18	13,01	5,72	7,29	0,008
Docetaxel	Tubulin stabilizer	18	13,88	8,95	4,93	0,033
Tofacitinib	JAK2/3	18	4,94	1,04	3,91	0,003
AZD1480	JAK1/2, FGFR	18	5,83	2,31	3,53	0,035
Momelotinib	JAK1/2	18		11,20	2,71	0,033
Doxorubicin	Topoisomerase II	17	5,36	7,54	-2,18	0,047
Nutlin-3	Mdm-2	18	2,99	5,21	-2,22	0,021
Chloroquine	Antimalaria	18	7,63	9,85	-2,22	0,035
Canertinib	pan-ErbB	18	7,86	10,09	-2,23	0,014
Belinostat	HDAC	18	16,87	19,15	-2,28	0,049
Fludarabine	Purine analog	17	7,53	9,86	-2,33	0,020
Etoposide	Topoisomerase II	17	2,12	4,64	-2,52	0,033
Panobinostat	HDAC	18	20,33	22,93	-2,59	0,026
UNC0642	G9a/GLP	17	2,92	5,95	-3,03	0,026
Amonafide	Topoisomerase II	18	3,08	6,73	-3,66	0,004
AZ3146	Mps1	18	1,56	5,27	-3,71	0,026
Pictilisib	PI3K	18	7,82	11,65	-3,83	0,033
Mitoxantrone	Topoisomerase II	17	2,67	6,67	-4,00	0,026
Daunorubicin	Topoisomerase II	17	7,56	11,61	-4,05	0,003
Teniposide	Topoisomerase II	17	9,63	14,13	-4,50	0,047
Quisinostat	HDAC	18	16,90	21,66	-4,76	0,003
Navitoclax	BCL-2/BCL-XL	18	16,92	22,00	-5,08	0,020
Omacetaxine	Protein synthesis	18	25,11	30,23	-5,13	0,000
Cabozantinib	VEGFR2, MET, FLT3, TIE2, KIT, RET	18	0,92	6,11	-5,19	0,033
Valrubicin	Topoisomerase II	17	8,06	13,29	-5,23	0,003
Idarubicin	Topoisomerase II	18	10,53	15,79	-5,26	0,026
AZD7762	CHK1/2	18	5,27	12,31	-7,04	0,035
Sunitinib	Broad TK inhibitor	18	2,26	9,69	-7,44	0,026
Ponatinib	Broad TK inhibitor	18	5,16	12,66	-7,50	0,033
Quizartinib	FLT3	18	0,79	8,47	-7,67	0,035
Foretinib	Broad TK inhibitor	18	2,93	10,83	-7,90	0,033
Venetoclax	BCL-2	17	7,78	17,31	-9,52	0,008

The main drug classes, which were affected by the *ex vivo* screening conditions were tyrosine kinase inhibitors such as JAK inhibitors, topoisomerase II inhibitors and BCL-2 inhibitors. Most of these inhibitors were less potent at killing AML cells in the stroma-derived condition except the JAK inhibitors. Sensitivity to JAK inhibitors was enhanced across the AML samples in CM, which is in line with the soluble factor mediated JAK/STAT activation in AML. Moreover, samples harboring *FLT3*-ITD or *CCDC88C-PDGFRB* rearrangements were less sensitive to several TKIs targeting FLT3, VEGFR, PDGFR, ABL and KIT, when tested in CM (Table 2).

5.1.4 Overcoming stroma-mediated drug resistance

BCL-2 protein antagonists are a promising therapy option for the treatment of AML. Hence, we investigated the molecular mechanisms of CM-mediated resistance to BCL-2 inhibition (Figure 7A-B). First, we determined the effects of different cell culture conditions on the expression of pro-survival factors, and measured the expression of *BCL-2* family genes after 48 hours of incubation in MCM and CM conditions. Cytokines contained in the CM decreased the expression of *BCL-2* in AML cells, whereas *BCL-XL* expression was upregulated. This indicates that the factors secreted by stromal cells lead to downregulation of the target molecule of BCL-2 antagonists. Furthermore, AML cell survival in CM was driven by the anti-apoptotic protein BCL-XL rather than BCL-2 (Figure 7C).

Subsequently, we wanted to better understand the impact of cytokines on drug resistance and tested the effect of abundant cytokines in CM to venetoclax response. Out of the tested cytokines, GM-CSF was identified to most effectively mimic the CM-induced resistance to BCL-2 inhibitor venetoclax (Figure 7D). Since GM-CSF is known to activate JAK/STAT signaling in leukemic cells[194,195], we further studied the impact of this signaling pathway on venetoclax resistance by knocking-down *STAT3* expression from a venetoclax resistant cell line. Increased sensitivity of *STAT3* knockdown cells to venetoclax validated the potential contribution of JAK/STAT signaling to venetoclax resistance.

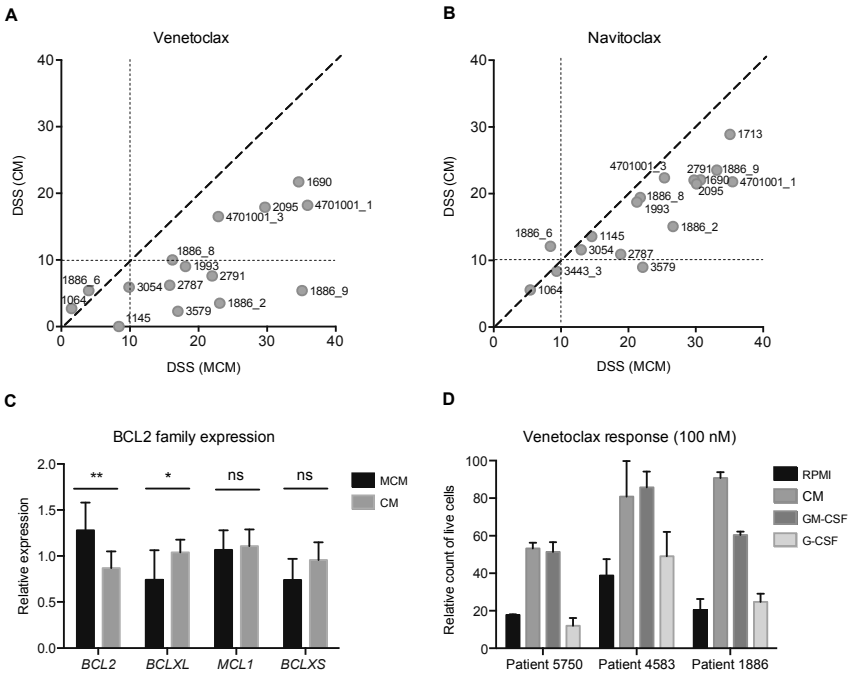

Figure 7. Stroma-based conditions reduce AML cell response to BCL-2 inhibitors. (A-B) AML patient samples show decreased sensitivity to BCL-2-specific inhibitor venetoclax and to BCL-2/BCL-XL inhibitor navitoclax in the HS-5 CM condition. (C) AML cell culturing in CM results in decreased *BCL2* expression and induction of *BCLXL* expression in the cells. Bar plots represent the mRNA expression for *BCL-2* genes after 48 hours of incubation of AML cells (n = 6) in 25% HS-5 CM and MCM medium. Data are normalized against GAPDH expression and error bars represent standard deviation of at least two replicates. (D) Effect of AML cell culturing in RPMI, 25% HS-5 CM, or RPMI supplemented with 10 ng/mL GM-CSF or G-CSF on venetoclax response. The amount of live $CD45^{+}$AML cells was analyzed by flow cytometry after 48 hours treatment with 100 nM venetoclax. Error bars represent standard deviation of three replicates. ns, not significant. $*P < .05$; $**P < .01$.

To follow up on the findings, we tested the ability of JAK1/2 inhibitor ruxolitinib to overcome stroma-induced resistance of AML cells to BCL-2 inhibition. Combination of venetoclax with ruxolitinib was synergistic in AML patient cells in CM and in a co-culture setting, either with direct contact to patient derived MSCs or separated with a pore membrane (Figure 8A-B). In contrast, inhibition

of JAK/STAT signaling was insufficient to overcome stroma-mediated resistance to FLT3 inhibitor quizartinib and no synergy was detected between ruxolitinib and venetoclax in the CM or MCM condition.

As AML cells became resistant to BCL-2 mediated cell death when separated from stromal cells, this indicates that the stroma-derived soluble factors are adequate to cause venetoclax resistance. Mechanistically, the combination of venetoclax with a JAK1/2 inhibitor ruxolitinib downregulated expression of the anti-apoptotic proteins BCL-XL, BCL-2 and MCL-1 (Figure 8C).

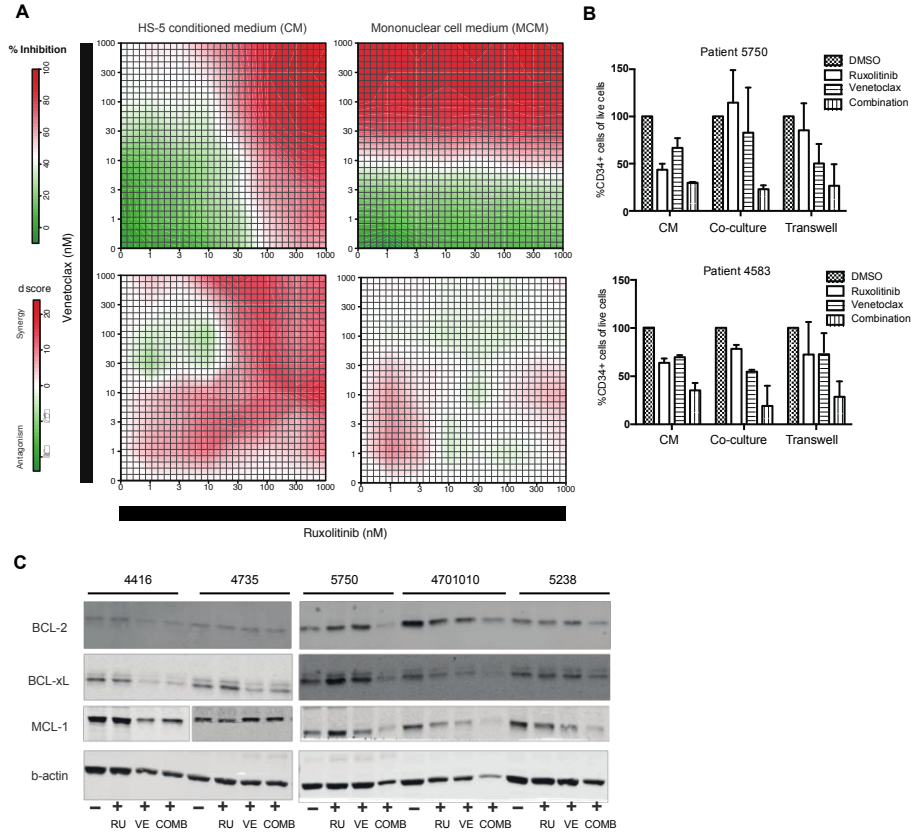

Figure 8. JAK1/2 inhibitor ruxolitinib restores activity of BCL-2 selective inhibitor venetoclax against AML patient cells in stroma-based conditions. (A) Combinatorial treatment of AML cells with ruxolitinib and venetoclax is synergistic in 25% CM. Eight concentrations of the drugs were tested in the two conditions for 72 h after which cell viability was measured with the CTG assay and dose-response matrices of percent inhibition were generated for the indicated concentrations. Dose-response matrices of

delta synergy scores were calculated with the ZIP model. δ, difference in %- inhibition compared with expected additive effect from the compound. (B) Amount of CD34+ AML cells after 48 h drug treatment with the indicated drugs (300 nM ruxolitinib, 100 nM venetoclax, or their combination) in RPMI, 25% HS-5 CM or with mesenchymal stem cells (MSCs) in a co-culture or a transwell setting, separated from stroma by a membrane with pore size of 0.4 μm. Error bars indicate standard deviation from two experiments. (C) Protein levels of BCL-2 family members in AML patient cells after treatment with 300 nM ruxolitinib (RU), 100 nM venetoclax (VE) or a combination of both drugs (COMB) in 25% HS-5 CM for 48 h. Membranes were visualized with the Odyssey imaging system.

Finally, we evaluated the potency of venetoclax and ruxolitinib combination to target AML cells in a xenograft mouse model. NSG mice were inoculated with MOLM-13^{luc} AML cell line expressing a luciferase marker. After leukemia engraftment, the mice were treated with venetoclax, ruxolitinib and a combination of both drugs in consecutive groups. Effects of the drugs on tumor burden were analyzed by bioluminescent imaging throughout the treatment period of three weeks, and one week after the treatment termination. At week four, the mice treated with the drug combination had a significant clearance of their leukemia burden, compared to single drug treated mice (Figure 9A-B). However, no improvement in the overall survival of the combination treated mice was accomplished due to toxicity issues.

Figure 9. Combined blockade of JAK/STAT and BCL-2 pathways with ruxolitinib and venetoclax is synergistic in an AML xenograft mouse model. (A) Bioluminescent images of 24 NSG mice inoculated with luciferase expressing MOLM-13 cells that were treated with vehicle, venetoclax (25 mg/kg, i.p.), ruxolitinib (50 mg/kg, p.o) or both drugs for three weeks. Images were taken before starting the treatments (week 0), at weeks 1-3 and one week after the treatments. (B) Mean bioluminescence recorded for each group at matching time points with panel A. Error bars represent standard error of the mean. $^{**}P<0.01$, by Mann-Whitney test.

5.2 Identification of biomarkers for BCL-2 inhibitor resistance (II)

Mechanisms and predictors of BCL-2 inhibitor resistance remain poorly understood even though most patients ultimately develop resistance to BCL-2 inhibitor treatment. In study II, we used a machine learning method to integrate RNA sequencing results with *ex vivo* DSRT data for BCL-2 inhibitor venetoclax, in order to identify biomarkers associated with venetoclax resistance. In addition, we investigated potential combination strategies to overcome venetoclax resistance in AML patient samples.

5.2.1 Molecular features of venetoclax-resistant AML patient samples

To identify biomarkers for venetoclax resistance, we performed a linear regression analysis between gene expression and *ex vivo* venetoclax responses of AML patient samples. This analysis resulted in 601 differentially expressed genes, which significantly associated with venetoclax response (FDR < 0.05). Of these genes, 252 (41.9%) were positively and 349 (58.1%) negatively associated with venetoclax response. Genes with the highest positive and negative association with venetoclax response are shown in Table 3. Venetoclax sensitivity associated positively with five *HOX* family genes (*HOXB5*, *HOXB6*, *HOXB7*, *HOXB8* and *HOXB9*). This finding is in line with a previous study by Kontro *et al,* who found *HOX* expression to predict response to BCL-2 inhibition[196]. Furthermore, venetoclax resistance was associated with a sub-cluster of 29 genes including three *S100* family genes (*S100A6*, *S100A8*, and *S100A9*) that were among the most significantly associated genes (Figure 10). Interestingly, S100A8 and S100A9 are abundant proteins in myeloid cells[197] and deregulation of these genes has been linked to progression of many tumor types. Pathway and network analysis of venetoclax resistance-associated genes using Enrichr analysis tool and Ingenuity Pathway Analysis showed many of them to be involved in function of the immune system and inflammation related responses. Interestingly, the *S100* family genes *S100A8* and *S100A9* are also known to stimulate inflammatory responses and the production of cytokines[198,199].

Table 3. *List of genes with the highest positive and negative association with venetoclax response (FDR < 0.05).*

HIGHEST POSITIVE ASSOCIATION				
Gene name	Fold change (log_2)	Average expression	P-value	adj. P-value
HOXB9	0,230895634	-0,5630024	7,29E-05	0,01370862
HOXB8	0,208259884	-2,1508601	7,73E-05	0,01370862
HOXB6	0,162296426	1,79894795	0,00133111	0,03900134
BEND6	0,157313606	0,98273025	1,15E-06	0,00144925
FGF10	0,155394614	-3,604052	0,00147455	0,04068075
HOXB5	0,139777143	0,24462293	0,00199805	0,04589318
NEK10	0,131918155	1,33297499	0,00023522	0,02000502
SPINK2	0,12579511	4,03385811	0,00221478	0,04848037
HOXB7	0,117613084	-0,0900622	0,00185345	0,0439348
MAMDC2	0,115312601	3,73650706	0,00040041	0,02495048
DST	0,093132657	6,03682686	0,00128783	0,0383214
WT1	0,089353945	2,59938381	0,00184551	0,04392958
RASSF8	0,07550781	2,53779151	0,00078285	0,03265667
SULT1C4	0,073229779	1,81123276	0,00114446	0,03712706
FLT3	0,071766075	8,17642162	0,00090646	0,03436618
RHOBTB1	0,071446448	3,60954454	0,00064464	0,02994137
SCD5	0,069564151	2,75645708	0,00077833	0,03265667
COL24A1	0,068920722	5,53448001	0,00031681	0,02249669
MSI2	0,068393534	7,77198342	0,00020243	0,01907017
IRGM	0,0636213	1,71969033	0,00029925	0,02189941
HIGHEST NEGATIVE ASSOCIATION				
CMTM5	-0,189763303	-1,0881435	0,00016718	0,0184975
NFATC4	-0,156212981	-2,5349857	0,00103499	0,03540044
NFIB	-0,150888586	-1,4106097	0,00156315	0,04101057
EPPK1	-0,116444165	-0,1746154	0,00055435	0,02801968
FCN1	-0,114642982	7,30275619	0,00012985	0,01738763
PLBD1	-0,111085537	5,07154633	0,00171306	0,04196649
ADM	-0,111080178	1,88437456	0,00231486	0,04930133
QPCT	-0,109300097	2,40956148	0,00093683	0,03476507
FPR3	-0,108781114	0,46757892	0,00235095	0,04959289
C5AR1	-0,10854503	4,11062641	0,00223052	0,0484897
VSTM1	-0,106950843	2,2834733	0,00025486	0,02083079
SLC11A1	-0,106845245	4,28187867	0,00050002	0,0273639
HK3	-0,104555137	5,60383777	0,00079705	0,03283197
S100A9	-0,102862649	9,1781141	0,00056786	0,02823409
SLC15A3	-0,101779214	2,50755844	0,00122558	0,03760769
DLEU7	-0,101085099	0,10857368	2,35E-05	0,00994287
S100A8	-0,099825883	8,44301442	0,00155839	0,04101057
CPAMD8	-0,097142788	0,28517741	0,00113637	0,03712706
FFAR2	-0,096801806	1,18349162	0,00208094	0,0470247
NCF1	-0,096111624	4,11443888	0,00084509	0,03332352

Subsequently, we investigated association of venetoclax resistance and gene expression with somatic, nonsynonymous mutations. This analysis revealed a co-occurrence of *S100A8* and *S100A9* overexpression and *TET2* mutations with venetoclax resistance (Figure 10).

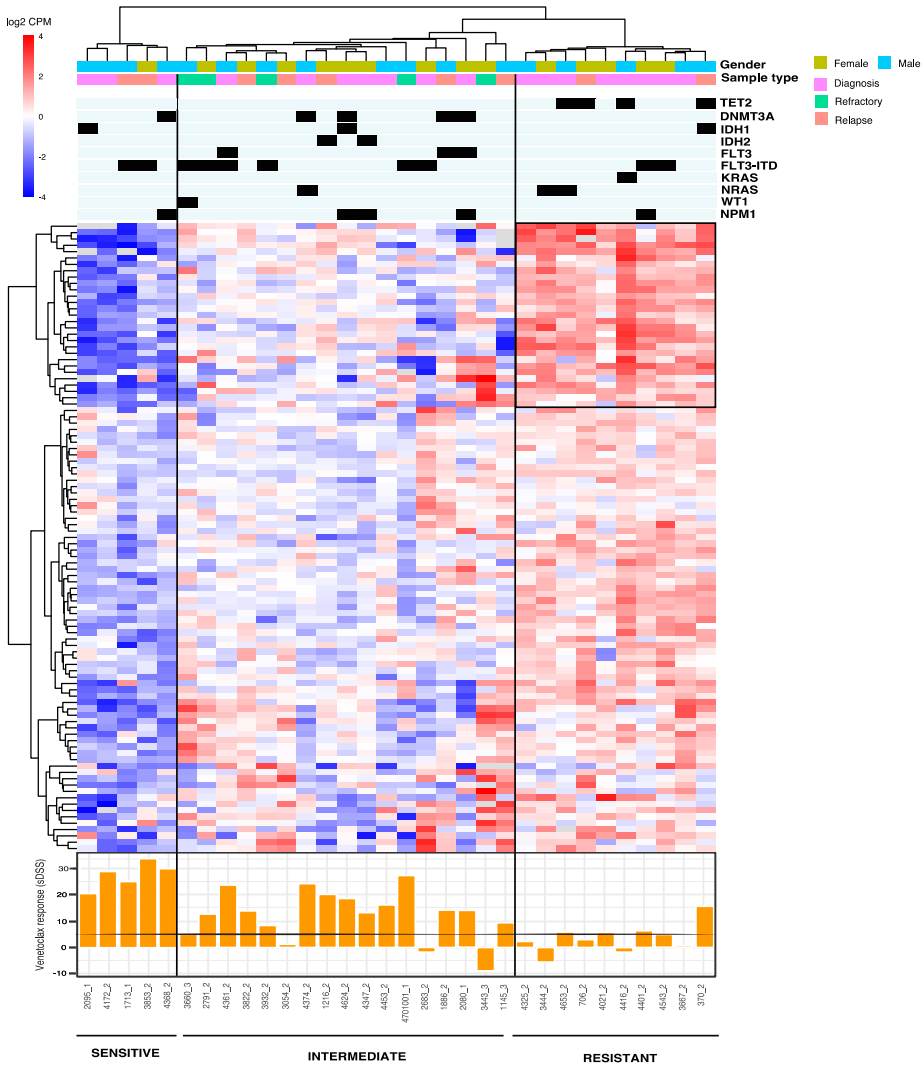

Figure 10. Molecular features of AML patient samples resistant to BCL-2 inhibitor venetoclax. Somatic mutations, mRNA expression and *ex vivo* venetoclax response of 32 AML patient samples. Top 100 significant genes associated with venetoclax resistance were clustered. Hierarchical clustering of samples and genes (mean centered $\log_2$ CPM)

was done using Euclidean distance matrix and complete clustering method. One of the major gene clusters consists of 29 highly upregulated genes (enclosed box) that strongly associate with venetoclax-resistance. Clustering of samples depicts three main groups. The bar plot displays venetoclax response (sDSS, selective drug sensitivity score) in matching AML patient samples in comparison to healthy donor samples. sDSS >5 indicates AML specific drug sensitivity.

5.2.2 Elevated expression of *S100A8* and *S100A9* correlates with venetoclax resistance

Expression of *S100* family genes is known to be deregulated in various tumor types[200-203] including AML. Intriguingly, high expression of S100A8 predicts poor survival in AML patients[204]. For these reasons, we were interested in the role of *S100A8* and *S100A9* as potential biomarkers for venetoclax resistance in AML. Analysis of the prevalence of mRNA expression of *S100A8* and *S100A9* in relation to other *S100* family genes confirmed the highest expression of *S100A8, S100A9*, *S100A4* and *S100A6* in a cohort of 112 AML patient samples. Furthermore, this finding was supported by The Cancer Genome Atlas (TCGA) AML dataset[1], in which 178 AML samples were divided into seven groups based on RNA expression profiles. Interestingly, one of the groups was detected to overexpress *S100A8* and *S100A9* along with other genes. This suggests that *S100* family gene expression might be a marker for a specific gene expression signature that predicts higher likelihood of developing resistance to venetoclax treatment.

To better understand the role of *S100* family genes in venetoclax response, we assessed sensitivity of six AML cell lines to venetoclax after 72 hours exposure to the drug. Cell lines that were resistant to venetoclax, namely NOMO-1, SKM-1 and SHI-1, displayed significantly upregulated expression of S100A8 and S100A9, whereas no expression was detected in the highly sensitive cell lines MOLM-13, Kasumi-1 and ML-2 (Figure 11A-B). To investigate potential mechanisms of S100A8/A9 mediated resistance to venetoclax, we analyzed the impact of high *S100A8* and *S100A9* expression on calcium release, as the S100A8/S100A9 complex is known to bind cytosolic calcium[205,206]. Calcium uptake by mitochondria promotes apoptosis by opening the permeability transition pore that leads to depolarization of the membrane and subsequent release of cytochrome c[207]. Previously, Spijkers-Hagelstein *et al* reported elevated

expression of S100A8 and S100A9 heterodimer to cause glucocorticoid resistance in MLL rearranged infant ALL cells[208]. Hence, we investigated the role of calcium binding on venetoclax resistance, and measured the amount of free-cytosolic calcium in venetoclax resistant and sensitive cell lines after 48 hours exposure to venetoclax. In the resistant cell lines, venetoclax-induced calcium release was lower compared to the sensitive cell lines, possibly impacting mitochondrial membrane permeabilization and induction of apoptosis (Figure 11C). Subsequently, we knocked-down *S100A8* and *S100A9* expression in venetoclax-resistant cell lines by CRISPR/Cas9 genome editing technology. As deletion of *S100A8* and *S100A9* expression did not rescue venetoclax resistance, we concluded calcium binding to be insufficient to directly cause resistance to BCL-2 inhibition. Thus, elevated expression of *S100A8* and *S100A9* genes can potentially serve as a prognostic marker or predictor of venetoclax resistance, but may not be a causal factor in the resistance. To elaborate the functional consequences of this finding requires further exploration.

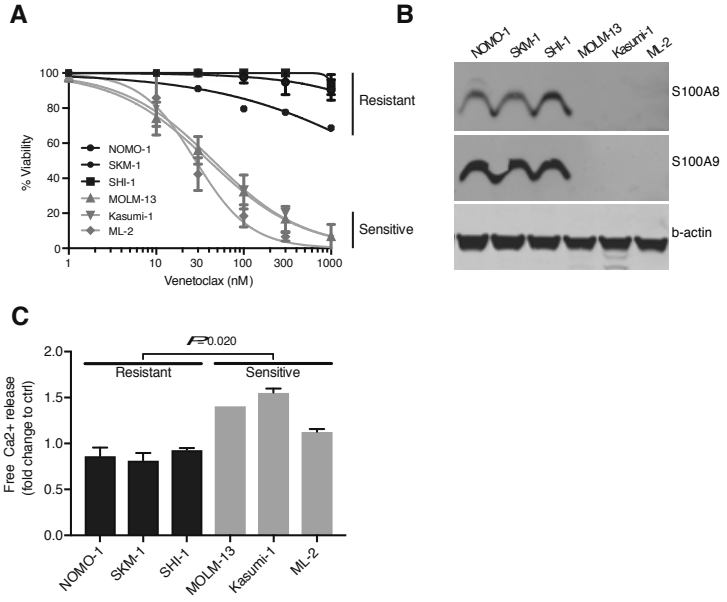

Figure 11. *S100A8* and *S100A9* have a high expression in venetoclax-resistant cell lines compared with sensitive cell lines. (A) Dose response curves of AML cell lines after treatment with indicated concentrations of venetoclax for 72 h. Cell viability was measured using the CTG assay. (B) Western blot analysis of S100A8 and S100A9 proteins in venetoclax-sensitive (Kasumi-1, MOLM-13, ML-2) and venetoclax-resistant (NOMO-1, SKM-1, SHI-1) cell lines. (C) Over-expression of *S100A8* and *S100A9* in venetoclax-

resistant cell lines is associated with failure to release free-cytosolic Ca^{2+} after exposure to venetoclax (100 nM) for 4 h. Difference in the calcium release was statistically analyzed using the Mann-Whitney *U*-test.

5.2.3 BET bromodomain inhibitor treatment restores sensitivity to BCL-2 inhibition

In order to identify potential synergistic drug combinations that could overcome venetoclax resistance, we investigated correlation of *S100A8* and *S100A9* mRNA expression with response to 349 drugs for 35 AML patient samples. Integrated analysis showed significant positive correlation with *S100A9* expression and sensitivity to a BET bromodomain inhibitor OTX-015 (Figure 12A). Subsequently, venetoclax was tested in combination with OTX-015 for AML cell lines and patient samples. Venetoclax resistant cell lines NOMO-1 and SKM-1, which express high levels of S100A8 and S100A9, reacted in a synergistic fashion to the combination treatment. Efficacy of the venetoclax/OTX-015 combination also correlated with the expression levels of *S100A8* and *S100A9* in primary AML patient samples. Seven samples (7/11) with high expression levels of *S100A8* and *S100A9* were sensitive to the drug combination, whereas in three out of four low expression samples, no synergy was observed (Figure 12B). These data demonstrate that OTX-015 confers or restores sensitivity to venetoclax in AML samples overexpressing *S100A8* and *S100A9*. Mechanistically, the OTX-015 treatment led to downregulation of BCL-2 and BCL-XL in the resistant cell lines, whereas BCL-XL was downregulated in some patient samples and BIM (encoded by *BCL2L11*) upregulated in others (Figure 12C).

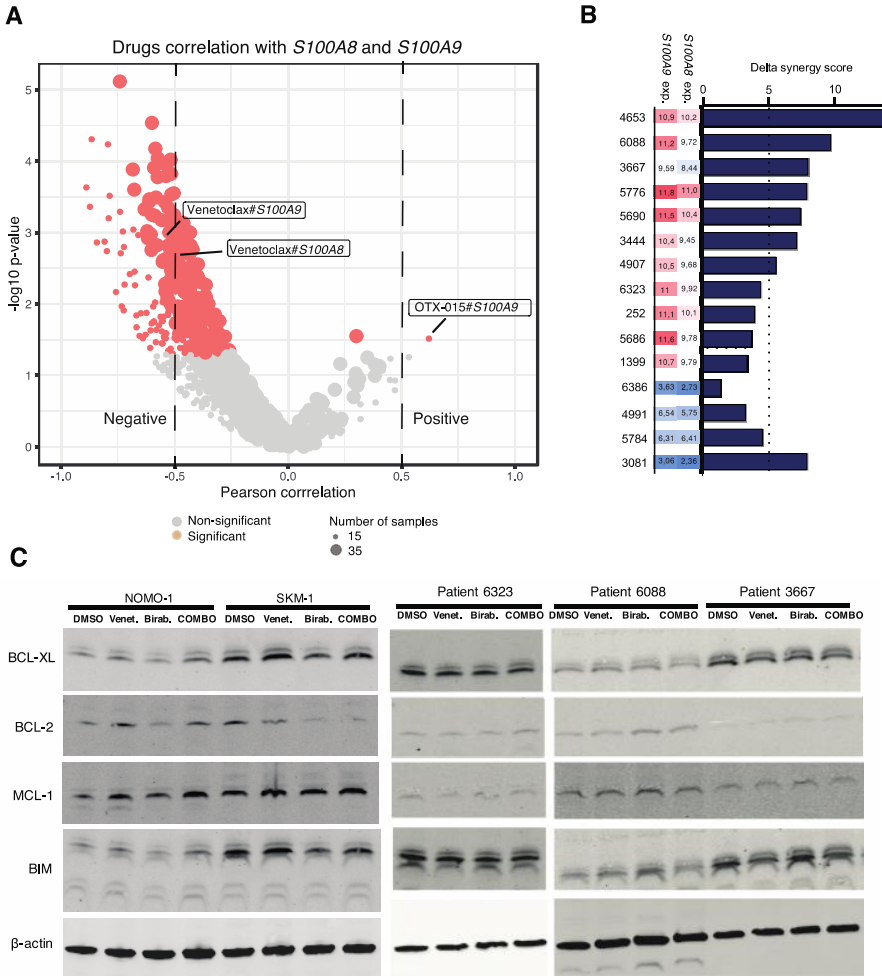

Figure 12. BET bromodomain inhibitor OTX-015 restores venetoclax sensitivity in cells lines and AML patient samples that overexpress *S100A8* and *S100A9*. (A) Drugs that show positive and negative correlation with the mRNA expression of *S100A8* and *S100A9*. Dot size indicates the number of tested AML patient samples whereas color indicates significance of the correlation. (B) Delta synergy scores for the tested drug combination in AML patient samples expressing high or low-level of *S100A8* and *S100A9*. Gene expression presented as $\log_2$ CPM values. A delta score > 5 indicates synergy. (C) Representative blots showing BCL-2 family proteins in AML cell lines and patient samples upon treatment with 100 nM venetoclax, 100 nM OTX-015 or their combination for 48 h.

5.3 Statistical evaluation of proteomic and transcriptomic profiles for biomarker discovery (III)

In study III, our aim was to compare the value of protein level versus mRNA transcript level detections for AML biomarker discovery. By comparing matching label-free LC-MS/MS and gene expression microarray measurements from four AML patient and six CD34+ healthy control samples from the study of Foss *et al*[182], we detected significant differences in the biomarker expression panels between the datasets (Figure 13). Proteome analysis revealed unique links to leukemic processes, demonstrating the value of label-free proteomic approaches for biomarker discovery in AML.

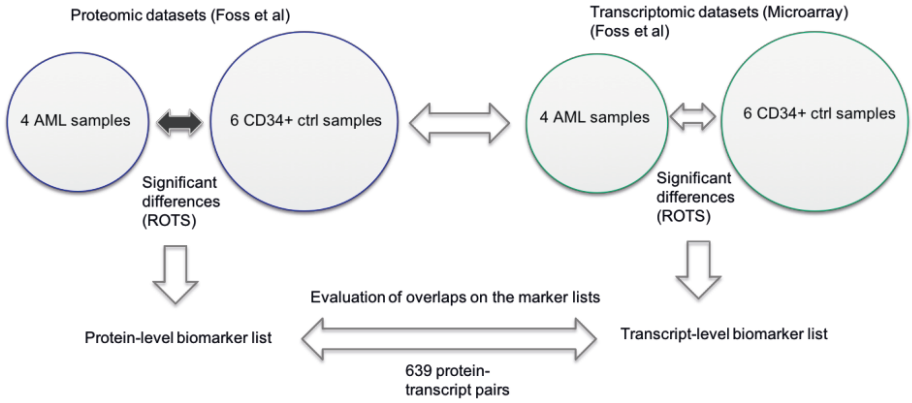

Figure 13. Study design used for comparing proteomic and transcriptomic datasets.

5.3.1 Proteomic and transcriptomic dataset detections

Reproducibility Optimized Test Statistic (ROTS) analysis of differential expression between the AML patient and control samples revealed 61 significant (FDR < 0.05) detections (indicating positive correlation) from the proteomic dataset and 30 from the transcriptomic dataset for 639 matched protein-transcript pairs. Twenty-three of the detections were shared between the assays, representing an enrichment of upregulated detections (Figure 14). Detections from the protein dataset showed a wide range of up- and downregulated expression changes capturing subtle differences whereas in the transcriptomic data the expression levels were mainly strongly downregulated.

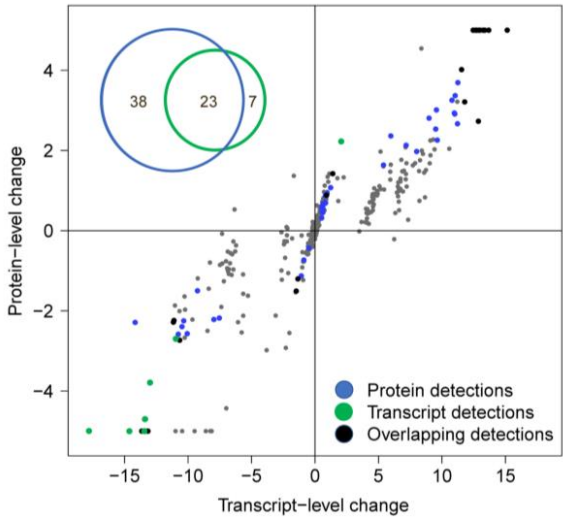

Figure 14. Patterns of protein and transcriptomic level detections. Average signal log ratios between four AML and six control samples for 639 matched protein-transcript pairs. Blue and red dots represent significant differential (FDR < 0.05) protein-level and transcript-level expression, respectively. Overlapping detections are marked in black. These detections are visualized with the Venn diagram. *Reproduced with the permission from Wiley publishing group*[209].

5.3.2 Links to hematopoietic diseases

In order to evaluate the relevance of the detections as potential biomarkers for AML, we classified the significant detections from both datasets based on known links to hematopoietic diseases. From the 38 protein specific detections, nine proteins were involved in different leukemic processes. The transcriptomic detections, however, were mainly linked to disease development in general, except for one leukemia specific detection. Out of 23 detections common between the datasets, two were linked to acute leukemias (Table 4).

Table 4. *Disease links for unique and shared protein and transcript-level detections (FDR < 0.05).*

Gene and protein identifiers		Disease links (manual classification)		
Gene ID	Protein name		Protein FDR	Transcript FDR
UNIQUE PROTEIN DETECTIONS				
ACO2	Aconitate hydratase mitochondrial		0.036	0.085
ACTN4	Alpha actinin 4	B: ovarian adenocarcinoma	0.000	0.091
CANX	Calnexin		0.029	0.086
CALR	Calreticulin	A: expressed in cancers including AML	0.000	0.079
CAT	Catalase EC 1 11 1 6	C: protects against oxidative damage	0.021	0.097
C1QBP	Complement component 1 Q subcomponent binding protein mitochondrial		0.036	0.130
CTSC	Dipeptidyl peptidase 1 EC 3 4 14 1		0.047	0.314
RAD50	DNA repair protein RAD50	C: DNA damage repair	0.038	0.071
RPN2	Dolichyl diphosphooligosaccharide protein glycosyltransferase subunit 2 EC 2 4 1 119		0.028	0.077
EPHB3	Ephrin type B receptor 3 EC 2 7 10 1	A: pre-B leukemic cell adhesion	0.020	0.065
ALDOA	Fructose bisphosphate aldolase A EC 4 1 2 13		0.020	0.138
FH	Fumarate hydratase mitochondrial	B: tumor suppressor	0.022	0.068
GSN	Gelsolin	B: colon adenocarcinoma and melanoma cell migration	0.023	0.263
PRKCSH	Glucosidase 2 subunit beta	B: polycystic kidney disease	0.024	0.279
HSPA8	Heat shock cognate 71 kDa protein		0.048	0.137
HCLS1	Hematopoietic lineage cell specific protein	A: expressed in early myeloid and erythroid lineage cells	0.022	0.239
H1F0	Histone H1		0.036	0.070
CAPG	Macrophage capping protein	B: Cholangiocarcinoma biomarker	0.000	0.132
NAT10	N acetyltransferase 10 EC 2 3 1	C: protects against DNA damage	0.038	0.136
GANAB	Neutral alpha glucosidase AB EC 3 2 1 84		0.029	0.088
SLC25A3	Phosphate carrier protein mitochondrial		0.033	0.093
PEBP1	Phosphatidylethanolamine binding protein 1		0.046	0.129
INPP5D	Phosphatidylinositol 3 4 5 trisphosphate 5 phosphatase 1 EC 3 1 3 n1	A: negative regulator of myeloid cell proliferation	0.027	0.098
GP1BA	Platelet glycoprotein Ib alpha chain	A: associated with thrombocytopenia	0.000	0.106
DDX6	Probable ATP dependent RNA helicase DDX6 EC 3 6 1	A: involved in the t(11;14) translocation in B-cell lymphomas	0.034	0.075
PA2G4	Proliferation associated protein 2G4	A: linked to CML signaling	0.024	0.091
SET	Protein SET	A: associated with myeloid leukemia	0.030	0.095
PDHB	Pyruvate dehydrogenase E1 component subunit beta mitochondrial		0.000	0.089
G3BP1	Ras GTPase activating protein binding protein 1 EC 3 6 1		0.000	0.228
SAFB	Scaffold attachment factor B	B: breast tumorigenesis	0.031	0.081
SHMT2	Serine hydroxymethyltransferase mitochondrial		0.020	0.067
BAT1	Spliceosome RNA helicase BAT1 EC 3 6 1	B: rheumatoid arthritis	0.037	0.133
SMARCC2	SWI SNF complex subunit SMARCC2		0.021	0.075
TXNDC5	Thioredoxin domain containing protein 5		0.000	0.086
TOR1AIP1	Torsin 1A interacting protein 1		0.000	0.083
TRIM28	Transcription intermediary factor 1 beta		0.000	0.092
TRA2A	Transformer 2 protein homolog		0.049	0.088
WAS	Wiskott Aldrich syndrome protein	A: B-cell development and function	0.048	0.128
UNIQUE TRANSCRIPT DETECTIONS				
CLIC1	Chloride intracellular channel protein 1	B: glioma. hepatocarcinoma	0.157	0.037
CORO1A	Coronin 1A		0.058	0.000
DLST	Dihydrolipoyllysine residue succinyltransferase component of 2 oxoglutarate dehydrogenase complex mitochondrial EC 2 3 1 61		0.083	0.000
NNT	NAD P transhydrogenase mitochondrial EC 1 6 1 2	C: protects against oxidative damage	0.104	0.000
DEFA1	Neutrophil defensin	C: increased expression in inflammatory conditions and cancer	0.084	0.000

PRMT1	Protein arginine N methyltransferase 1 EC 2 1 1	A: increased expression associated with acute lymphoblastic leukemia	0.248	0.033
STRADA	STE20 related adapter protein	C: binds and activates LKB1	0.069	0.000
COMMON DETECTIONS				
ACAA2	3 ketoacyl CoA thiolase mitochondrial EC 2 3 1 16 *	C: deficiency associated with netropenia and thrombocytopenias	0.000	0.000
ABHD14B	Abhydrolase domain containing protein 14B EC 3		0.000	0.000
ATP5J2	ATP synthase subunit f mitochondrial		0.000	0.000
ATP5O	ATP synthase subunit O mitochondrial		0.025	0.038
DDB1	DNA damage binding protein 1	C: responsible for UV DNA damage repair	0.023	0.034
GPX1	Glutathione peroxidase 1 EC 1 11 1 9	C: protects against oxidative DNA damage	0.026	0.000
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	B: pancreatic cancer	0.026	0.000
LDHA	L lactate dehydrogenase A chain		0.000	0.000
CYB5R3	NADH cytochrome b5 reductase 3		0.000	0.000
NCSTN	Nicastrin *	B: cancers and Alzheimer's disease	0.000	0.000
PSIP1	PC4 and SFRS1 interacting protein	A: found to be fused to NUP98 in one case of ANLL	0.000	0.000
PRDX1	Peroxiredoxin	C: protects against oxidative damage	0.000	0.000
PRDX2	Peroxiredoxin 2 EC 1 11 1 15	C: protects against oxidative damage	0.032	0.036
PAFAH1B2	Platelet activating factor acetylhydrolase IB subunit beta EC 3 1 1 47		0.000	0.000
ALDH1A1	Retinal dehydrogenase 1 *	A: involved in retinoic acid biosynthesis and AML	0.000	0.000
PRSS57	Serine protease 1 like protein 1 EC 3 4 21		0.000	0.000
SOD2	Superoxide dismutase Mn mitochondrial EC 1 15 1 1	B: hepatocellular carcinoma	0.000	0.000
TCP1	T complex protein 1 subunit alpha		0.000	0.000
CCT6A	T complex protein 1 subunit zeta		0.000	0.000
TMED10	Transmembrane emp24 domain containing protein 10	B: Alzheimer's disease	0.000	0.000
TMEM173	Transmembrane protein 173	C: activates NF-kB pathway	0.000	0.000
OCSTAMP	Transmembrane protein C20orf123		0.000	0.000
HADHA	Trifunctional enzyme subunit alpha mitochondrial		0.000	0.000

Significant detections were classified into three classes: A. linked to hematopoietic diseases; B. involved in other types of cancers and other diseases; C. linked to disease development in general. *. "all or none" biomarkers identified in the original study by Foss *et al*. False discovery rate (FDR) for the optimized ROTS statistic was calculated by randomly permuting the sample labels.

Furthermore, we validated the expression of three proteins glutathione peroxidase 1 (GPX1), fumarate hydratase (FH) and DEAD-box helicase 6 (DDX6) by Western blotting in a set of AML patient samples and one healthy control sample. The antioxidant enzyme GPX1 and tumor suppressor FH, which had not been previously linked to leukemia, were overexpressed in the tested AML patient samples compared to the healthy control sample validating the findings of Foss *et al* study (Figure 15A). The protein level of DDX6, however, was inconsistent between the AML samples in line with the known heterogeneity of AML patients. Further pathway analysis of the proteins and the significant detections from the proteomic and transcriptomic datasets with the Ingenuity Pathway Analysis

connected them to PI3K and ERK1/2 signalling proteins that are part of key dysregulated pathways in cancer (Figure 15B).

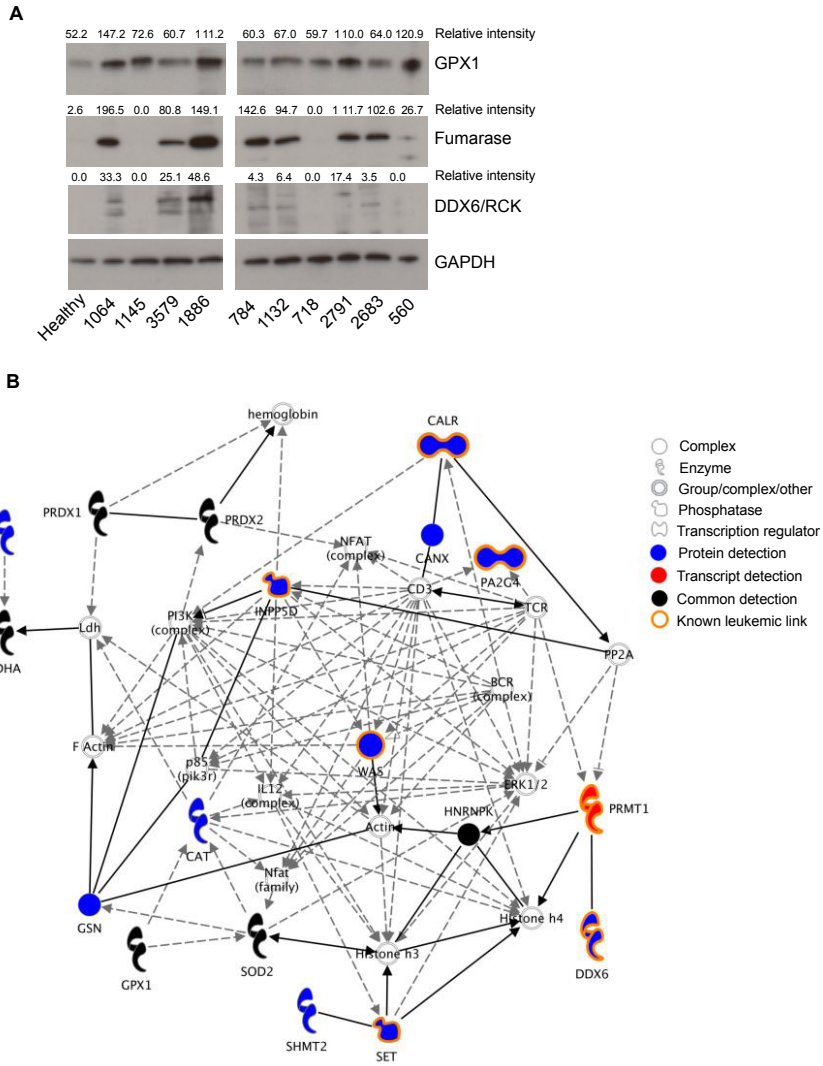

Figure 15. Western blot analysis of three selected protein level detections and their connection to signalling pathways. (A) Western blot analysis of the indicated proteins in 10 AML patient samples and one healthy donor sample. Intensity shown relative to integrated intensity of GAPDH. (B) Network analysis of the significant detections between AML and control samples constructed using Ingenuity Pathway Analysis software. Solid and dotted lines represent direct and indirect interactions, respectively. *Reproduced with the permission from Wiley publishing group*[209].

6 DISCUSSION

Genomic profiling has inspired the development of novel targeted therapies for AML. Targeting specific genomic alterations, however, has proven less successful than envisioned, mainly due to the development of drug resistance, which leads to clonal expansion of drug resistant clones and subsequent relapse of the disease. Hence, further understanding of the pathophysiological mechanisms of drug resistance is needed for finding personalized treatment options for AML.

New technologies such as high-throughput proteomics and drug screening could facilitate the discovery of biomarkers and indicators of drug resistance to targeted therapies. In this thesis, we investigated stroma-mediated drug resistance mechanisms and protein biomarkers for AML. We demonstrate that stroma-derived cytokines reduce BCL-2 inhibitor venetoclax mediated killing of AML cells. We provide evidence that inhibition of activated JAK/STAT signalling can re-sensitize venetoclax resistant AML patient cells and xenografts to venetoclax.

Furthermore, we have shown that expression of *S100A8* and *S100A9* genes predicts resistance to venetoclax. Finally, parallel analysis of proteome and transcriptome profiling data of AML patient samples led to discovery of AML-specific protein biomarkers.

6.1. BM microenvironment mediated drug resistance in AML

In study I, our objective was to facilitate personalized therapy of AML with *ex vivo* drug sensitivity and resistance testing (DSRT) method and thereby help the clinical translation of drug response data[210]. To meet this objective, we evaluated how stroma-derived soluble factors affect drug responses. We used BM-stroma derived medium to mimic conditions that are present in the BM, since the cancer microenvironment is known to protect leukemia cells from therapy-mediated cell killing. Our goal was to characterize microenvironment-mediated drug resistance across drug classes and to discover novel mechanisms of drug resistance.

These goals are particularly important, since most elderly patients with AML develop refractory disease and relapse. Moreover, traditional *ex vivo* culture

conditions insufficiently mimic the BM microenvironment, thereby the results from preclinical drug testing studies do not correlate with responses observed *in vivo*. We discovered that AML cells retain their viability better in the presence of stroma-derived growth factors, as compared to standard medium. Moreover, we found that *ex vivo* drug response to 12% (36/304) of the tested drugs was significantly different under exposure to BM stroma-derived medium.

Difficulties in culturing and maintaining AML cells *ex vivo*[211] restrict the accuracy of data derived from functional drug testing assays[212]. In our study, we used CM from the immortalized human stromal cell line HS-5 for testing *ex vivo* drug sensitivities of primary AML cells. Cytokine-enriched CM has been reported to induce the growth of myeloid colonies[191]. We discovered that stroma-derived growth factors induce resistance to inhibitors of topoisomerase II, BCL-2 and many tyrosine kinases (Table 2). Moreover, resistance to these inhibitors was undetectable in a standard MCM. Interestingly, the majority of AML patient samples developed resistance to conventional chemotherapeutic agents such as daunorubicin or idarubicin. However, another conventional chemotherapy agent, cytarabine, was not affected by the soluble stroma-secreted factors. Consistently, direct cell-to-cell contact between stromal cells and leukemic AML cells was shown to protect AML cells from cytarabine induced apoptosis[193]. In line with our findings, BM-derived soluble factors that are present in whole blood samples have also been shown to reduce the response of AML cells to topoisomerase II inhibitors[213].

Many receptor tyrosine kinase inhibitors, including FLT3 inhibitors, were less effective at killing AML cells in the presence of CM, as compared to standard medium. FLT3 inhibitors have been under active development for almost two decades[214,215], since *FLT3* is one of the most frequently mutated genes in AML and it associated with poor prognosis[110,216]. Responses to FLT3 inhibitors are often transient due to their inability to eliminate minimal residual disease (MRD). The BM microenvironment has been reported to play an important role in the survival of *FLT3* mutated AML cells undergoing FLT3 inhibitor treatment. Co-culture studies with stromal cells have indicated that soluble factors such as angiopoietins, GM-CSF, G-CSF, TNF and VEGF, IGF, EGF, and CXCL12 mediate the protective effect[138,217]. In line with these findings, stromal cells from quizartinib treated AML patients were showed to have increased expression of FGF2, which protected AML cells from FLT3 inhibition through activation of RAS/MAPK

signaling[136]. Interestingly, we detected high levels of GM-CSF and G-CSF in the HS-5 CM in our study, suggesting that these growth factors could potentially mediate FLT3 inhibitor resistance. In accordance, a recent study by Sung and colleagues reported GM-CSF and IL-3 to mediate resistance to FLT3 inhibitors[145]. Additional studies are needed to confirm potential relevance of GM-CSF for the development of FLT3 inhibitor resistance.

Since GM-CSF and G-CSF induce JAK/STAT signaling, we tested whether AML cells could be re-sensitized to FLT3 inhibitors by simultaneously blocking JAK-STAT signaling. The JAK1/2 inhibitor ruxolitinib, however, was unable to restore quizartinib sensitivity in the CM condition. This finding was inconsistent with earlier studies, which tested first generation FLT3 inhibitors with JAK inhibitors[137,218,219]. In addition to the JAK/STAT pathway, FLT3-ITD can also activate MAPK and PI3K/AKT signaling pathways [220], so it may be more relevant to target these pathways to overcome stroma-induced FLT3 inhibitor resistance. In support of this, Yang *et al* demonstrated that ERK activation, downstream of MAPK pathway, rather than STAT5 leads to quizartinib resistance in *FLT3*-ITD+ AML cell lines and patient cells[217]. Interestingly, these results were in line with our observations regarding the JAK1/2 inhibitor ruxolitinib. Hence, it appears that activation of STAT5 by the BM has an inconsistent role for the development of FLT3 inhibitor resistance[137].

Another drug class, which was ineffective in CM, was the BCL-2 inhibitors. Previous studies have shown that besides BCL-2 mutations, upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins may induce resistance to BCL-2 inhibition[77,221,222]. The role of extrinsic factors on BCL-2 inhibitor resistance in AML has not been fully characterized. We discovered that GM-CSF may confer venetoclax resistance in AML patient samples. Previous studies have shown that GM-CSF induces expression of the anti-apoptotic proteins BCL-2 and BCL-XL[223]. Consistently, *BCL-XL* expression was upregulated in AML cells cultured in CM, while *BCL-2* expression was downregulated. Thus, the shift to BCL-XL mediated cell survival in CM could explain the loss in sensitivity to the BCL-2 specific inhibitor venetoclax, which has only modest affinity to BCL-XL. Similar to GM-CSF, IL-6 was shown to induce BCL-XL and MCL-1 expression in multiple myeloma cells[224]. Gupta and colleagues showed that IL-6 causes resistance to venetoclax and to the dual BCL-2/BCL-XL inhibitor ABT-737 through redirecting the binding of pro-apoptotic BIM to MCL-1 instead of BCL-2[225]. We also tested the impact of IL-6 on

venetoclax sensitivity on AML cells, but concluded that IL-6 is insufficient at mediating the resistance alone. In the study of Gupta *et al*, MCL-1 dependency was reversed by targeting of JAK1/2 or MEK, downstream of IL-6 signaling pathways. Consistently, we found that JAK1/2 inhibitor ruxolitinib can restore *ex vivo* venetoclax sensitivity of AML cells in the CM and in a xenograft model of AML.

Kurtz *et al* reported several synergistic drug combinations with venetoclax using unfractionated BM cells, meaning that both blast cells and stroma-derived factors were present[226]. Importantly, they validated efficacy of venetoclax and ruxolitinib combination in a cohort of 195 samples consisting of newly diagnosed and relapsed/refractory AML samples[227]. Intriguingly, a phase I clinical trial that evaluates the safety and tolerability of venetoclax in combination with ruxolitinib in relapsed/refractory AML patient samples recently started enrolling patients (NCT038740529). Future work will show whether this combination shows efficacy *in vivo* and which drug resistance mechanism may emerge in these patients. Further studies are also warranted for discovering potential gene expression signatures that could predict patients' sensitivity to this combination.

Physiologically more relevant *ex vivo* and *in vivo* models are important for modeling interactions of leukemia cells with the BM microenvironment. Although CM, derived from a stromal cell line HS-5, improved viability of AML cells and promoted their emerging resistance to BCL-2 and FLT3 inhibitors, other interesting possibility would be to culture cells in the presence of the patient's own serum. This approach would also consider inter-patient heterogeneity. Similarly, mouse models using BM MNCs from patients rather than cell lines could potentially better maintain the critical features of cancer cells and their interactions with the microenvironment[228,229]. Although there has been some success in using patient derived xenografts (PDX) for identifying personalized treatment options[230], the long latency of disease initiation and the highly variable efficacy of engraftment rate are the most important, limiting challenges with these models. Moreover, differences in the murine versus human microenvironment, secretion of cytokines and growth factors may impact results obtained from mouse models[231]. Based on these factors, the results of our study also need to be interpreted carefully, as we tested human AML cells in a murine BM microenvironment.

6.2 Biomarkers of BCL-2 inhibitor resistance

The BCL-2 inhibitor venetoclax recently received FDA approval for the treatment of unfit AML patients in combination with hypomethylating agents or low dose cytarabine[115]. Despite this recent approval, there is little information regarding how patients will respond to this novel therapy. It is anticipated that most patients treated with venetoclax will eventually become resistant, which makes the identification of novel combinatorial treatments particularly important. In study II, our aim was to identify gene expression profiles or signatures linked to venetoclax sensitivity and resistance. Our analyses revealed that overexpression of *S100A8* and *S100A9* correlates strongly with venetoclax resistance. Moreover, AML patient samples with high *S100A9* expression showed significantly increased sensitivity to BET bromodomain inhibitor OTX-015, which in combination with BCL-2 inhibitor venetoclax was highly synergistic towards venetoclax-resistant AML patient cells.

Besides alterations in the expression of anti-apoptotic members such as BCL-XL and MCL-1, determinants of venetoclax sensitivity and resistance are being studied actively. Preclinical studies have suggested that AML cells with *WT1* and *IDH1/2* mutations have increased sensitivity to venetoclax[196,232]. The increased sensitivity of *IDH1/2* mutated samples to venetoclax resulted from *R*-(2)-hydroxygluatarate-mediated inhibition of cytochrome c oxidase that induced BCL-2 dependence[233]. Moreover, AML patients with *SRSF2* and *ZRSR2* mutations are more likely to respond to venetoclax, whereas patients with *PTPN11* or *FLT3*-ITD mutations are less responsive[233]. A study investigating biomarkers predicting venetoclax sensitivity from 200 AML patient samples found that samples harboring *KRAS*, *PTNPN11*, *SF3B1,* and *TET2* mutations are more resistant to venetoclax-mediated cell killing[234]. Interestingly, *TET2* mutations cause DNA hypermethylation[235] and have been connected with chemokine production and activation of inflammatory pathways[236,237]. Somatic mutation information from our cohort indicated that patients with *TET2* mutations show higher expression of *S100A8* and *S100A9*, and display reduced sensitivity to venetoclax, compared to patients with wild type *TET2*. Intriguingly, high expression of S100A8 and S100A9 has been suggested as a biomarker for inflammation[238], as these proteins are involved in innate and chronic inflammation[239]. Based on a recent study, p38 MAPK induces inflammatory signaling and survival of AML cells and inhibition of p38 in combination with venetoclax leads to enhanced cell killing[240]. Taken together, these data suggest

that an inflammatory response may be linked to venetoclax resistance. Future studies should further explore the role of inflammation and inflammatory pathways in venetoclax resistance.

In a subgroup of AML patients, *S100A8* and *S100A9* genes were found to be significantly overexpressed. This finding was supported by The Cancer Genome Atlas (TCGA) Research Network study, which included gene expression data from 178 AML samples[1]. Interestingly, three genes in addition to *S100A8* and *S100A9*, namely *PSAP, NAGA* and *CYBB*, were also upregulated in the venetoclax-resistant patient samples analyzed in our study. Moreover, *S100A8* and *S100A9* were also upregulated in venetoclax-resistant samples in a study by Kontro *et al,* who found that overexpression of *HOX* genes may predict venetoclax sensitivity[196]. Importantly, further analysis using data from the Beat AML collaboration validated significant negative correlation of *S100A8* and *S100A9* expression with venetoclax sensitivity in 186 AML patients[241]. This indicates that a subgroup of AMLs with a specific gene expression pattern might be more prone to venetoclax resistance.

Many preclinical studies are currently searching for optimal compounds that could be combined with venetoclax for durable drug responses. By applying machine learning, we found that *S100A9* expression correlates with high BET bromodomain inhibitor (OTX-015) sensitivity. Interestingly, OTX-015 in combination with venetoclax was highly synergistic in a subgroup of AML patient samples overexpressing *S100A8* and *S100A9*. Notably, Peirs *et al* found that the BET inhibitor JQ1 in combination with venetoclax synergizes in T-ALL xenograft models. Similar findings have been made in lymphoma[242-244] and AML[226]. Recently, another BET family bromodomain inhibitor (ABBV-075) was shown to modulate apoptotic pathways and to synergize with venetoclax in AML patient cells, cell lines and a xenograft mouse model[245,246]. As a result, this combination is currently under evaluation in a phase I clinical study (NCT02391480). Mechanistically, BET inhibitors have been shown to downregulate anti-apoptotic proteins BCL-XL, BCL-2 and MCL-1. In our study, we observed that OTX-015, which binds to bromodomain motifs BRD2-BRD4 of the BET proteins, decreased BCL-XL and BCL-2 level in two cell lines expressing *S100A8* and *S100A9*. In contrast, the AML patient samples had heterogenous expression of BCL-2 demonstrating that other factors besides BCL-2 may influence venetoclax responses. Moreover, OTX-015 treatment led to upregulation of pro-apoptotic

BIM, indicating that the BET bromodomain inhibition may induce apoptosis. In support of these findings, upregulation of BIM has been shown after JQ1 and ABBV-075 treatment in AML cell lines, T-ALL cells, and in other cancers[242,246-248].

Identification of patients that may benefit from investigational therapies such as venetoclax–OTX-015 combination remains challenging without robust biomarkers. In study II, we used gene expression profiling, *ex vivo* drug sensitivity data, and publicly available data sets to identify biomarkers of drug sensitivity and resistance. Further investigations, however, should be done with serial samples from venetoclax treated and clinically relapsed patients to validate our findings. Moreover, the predictive power of *ex vivo* drug testing should be evaluated more comprehensively, especially for epigenetic drugs such as OTX-015 that may require long periods of treatment before their efficacy can be accurately evaluated.

6.3 Quantitative protein biomarkers in AML

Improvements in proteomics approaches, such as label-free techniques and software tools have expanded the amount of information obtained from protein expression profiling in recent years[249-253]. However, the value of protein markers as opposed to DNA- and RNA markers for AML biomarker discovery is inadequately understood. Furthermore, experimental reproducibility and proper statistical testing remain challenges for protein biomarker studies[254]. In study III, our aim was to analyze how protein markers can bring novel insights into AML disease biology compared to markers derived from mRNA transcript profiling. The study was based on the re-analysis of matching proteomic and microarray datasets derived from AML and healthy control samples[182] using the bioinformatic "reproducibility optimized test statistic" (ROTS) approach[255,256].

Correct statistical procedures are needed for identifying clinically relevant biomarkers from highly heterogeneous patient material. In particular, analysis of proteomics data poses specific challenges as the assays are susceptible to miss protein identifications and suffer from unquantifiable, low protein levels. Statistical problems have led to deficient marker lists that are difficult to validate clinically. In comparison, appropriate data analysis and different technical nature of gene expression data have helped to generate reproducible results[257]. To

consider these points, we used the ROTS approach for ranking protein and transcript detections in order of differential expression. The ROTS method is more robust than conventional ranking methods for analyzing different datasets, as it learns an adjusted test statistic for each dataset based on inherent distributional properties. On the contrary, conventional ranking methods use the same, potentially sub-optimal statistic for each experimental assay.

The ROTS analyses resulted in 38 unique protein detections from the AML samples. Out of those, nine had connections to leukemia, in comparison to one transcript-level detection linked to hematological diseases. The original study of Foss and colleagues detected 17 differentially expressed proteins between AML patients and the control group. From those, retinal dehydrogenase, nicastrin, and 3-ketoacyl CoA thiolase were potential biomarkers for AML after excluding proteins below the detection level in either group[182]. These three biomarkers were also amongst the 23 common detections made in our study, expressed both on the mRNA and protein level. These results demonstrate that in-depth and quantitative statistical treatment of proteomic and transcriptomic data without excluding any low abundance proteins, provides an increased number of potential predictive biomarkers for leukemia. Furthermore, our analysis shows that proteomic profiling could yield more statistically significant and novel detections for AML in comparison to transcriptomic analysis.

Interestingly, one of the significantly upregulated proteins (FDR<0.05) was GPX1, which protects cells from oxidative stress[258]. Since GPX1 had not been linked to leukemia, we validated its expression using immunoblotting and found overexpression of GPX1 in AML cells compared to healthy cells. The finding supported the results from the bioinformatic ROTS analysis. A similar study by Pei *et al* detected overexpression of GPX1 in CD34+ AML cells compared to healthy CD34+ cells[259]. This validated our novel findings made with the ROTS procedure that were missed out with a regular *t*-test (FDR = 0.24).

The actual clinical value of protein markers for AML remains to be determined with different technologies and large patient cohorts. Currently, only a few relevant protein biomarkers have been discovered and verified clinically for AML. One such biomarker is the S100A8 protein that was discovered in a mass-spectrometry study of 54 AML patient samples. In this study, specific protein signatures were discovered for different cytogenetic groups. Of the detected

proteins, S100A8 was the most relevant protein, which was subsequently verified to reliably predict poor prognosis in AML with high specificity and sensitivity[204]. Future studies are needed for establishing larger, clinically relevant protein biomarker panels for AML. This could improve the utility of protein biomarkers for classifying and monitoring patients as well as discovering individually tailored treatment options.

7 CONCLUSIONS

In this thesis, we utilized high-throughput drug sensitivity and resistance (DSRT) testing assay to evaluate BM microenvironment mediated drug resistance in AML patient cells. In addition, we applied machine learning method for integration of RNA sequencing results with DSRT data to discover biomarkers associated with BCL-2 inhibitor resistance. Finally, ROTS-optimized statistics was used for deriving biomarker panels from proteomic and microarray transcriptomic assays and the results were compared in the context of AML dysregulated processes and networks.

The main findings of this study are:

1. BM stromal cells mediate protection of AML leading to BCL-2 inhibitor resistance, which can be overcome with the inhibition of JAK1/2.

2. Elevated expression of genes encoding for calcium-binding proteins S100A8 and S100A9 correlates with acquired resistance to the BCL-2 inhibitor venetoclax. In contrast, the BET inhibitor OTX-015 can reverse venetoclax resistance in AML patient samples overexpressing *S100A8* and *S100A9*.

3. Application of new statistical detection method for protein biomarker discovery provided unique and significant AML markers compared to microarray-based transcriptomic assay.

8 ACKNOWLEDGEMENTS

The thesis work was carried out at the Institute for Molecular Medicine Finland (FIMM), University of Helsinki. I am grateful for the chance to do my PhD in such an inspiring and dynamic research institute and to work with exceptionally passionate and focused people. I would like to sincerely thank all those who have helped me along the journey of my PhD.

Firstly, I would like to thank Doctoral School in Biomedicine (DPBM) for coordinating excellent training and financially supporting my studies. I am grateful for the opportunity to be in the DPBM student council (2013-2015) and to partake in the organization of the annual student symposium. I would like to express my gratitude to the Finnish Association of Hematology, Blood Disease Foundation, Cancer Society Finland, Orion Research Foundation, Instrumentarium Science Foundation, Oncology Society of Finland, Väre Foundation for Pediatric Cancer Research, K. Albin Johansson Foundation**,** Ida Montin Foundation and Maud Kuistila Memorial Foundation for the research grants during my doctoral studies.

I sincerely thank my supervisors Doctor Caroline Heckman and Professor Jonathan Knowles for the opportunity to do my doctoral studies at the Translational Research and Personalized Medicine group. Thank you, Caroline, for your guidance and support over the years. You have let me grow both professionally and personally. Thank you for your patience and encouragement. I admire your devotion for research and strategy in developing the Personalized medicine research in Finland and globally. I think you are exceptionally good at setting collaborations with the pharmaceutical industry. I thank Jonathan for being a great mentor. I am thankful for the meetings we had and highly value your advises regarding research and career in general. I have been privileged to have you as my co-supervisor and thank you for your time and inspiration in Personalized medicine.

I would like to acknowledge my thesis committee members Adjunct Professor Kaisa Lehti and Docent Matti Korhonen for following my progress and keeping me on track over the years. Thank you for your feedback and encouragement. My sincere appreciation goes to Adjunct Professor Urpu Salmenniemi and Adjunct Professor Matthias Nees for reviewing the thesis during their summer holidays

and providing constructive, yet positive feedback. Thank you, Doctor Eva Szegezdi, for accepting the honourable task of being my opponent.

It has been wonderful to be part of the Personalized Medicine (PM) group at FIMM. I am obliged to thank the principal investigators Professor Olli Kallioniemi, Professor Kimmo Porkka, Professor Krister Wennerberg, Professor Tero Aittokallio and Professor Satu Mustjoki for their leadership and visions. It has been fascinating to work together towards personalizing the treatment of leukemia. I thank all the colleagues in the PM group for discussions and for much appreciated help with my projects. Special thanks to the fellow PhD students in the group for your support and friendship.

I express my deep gratitude to all the co-authors for your contributions to the publications. Tea Pemovska, thank you for all your help and brainstorming with study I. I have truly admired your strength and determination since we started the PhD journey together. Thank you, Minxia Liu, for your contributions with study II. I would like to thank Ashwini Kumar for his bioinformatics expertise and Komal Kumar Javarappa for his help with flow cytometry experiments. Bhagwan Yadav, Liye He, Jing Tang and Tero Aittokallio are acknowledged for their contributions to drug sensitivity and combination synergy analysis. I thank Mika Kontro for providing patient material and clinical information.

I would like to acknowledge Siv Knaappila, Minna Suvela and Alun Parsons for taking care of sample processing and assisting with many technicalities in the lab. I am grateful to Alun for his expertise with RT-qPCR. Moreover, I would like to express my gratitude to the High Throughput Biomedicine Unit and the Sequencing Unit for your contributions. I thank our collaborators in Bergen for the opportunity to conduct mouse experiments in your facilities and would like to thank Bjørn Tore Gjertsen, Emmet McCormack, Michaela Popa and Mireia Mayoral Safont for the collaboration.

I thank my dear office mates Mamun, Heikki, Ashwini, Jarno and Dimitrios for your company and great times together. You have truly been amazing colleagues and have seen me through ups and downs. Your support, encouragement and friendship have been very important to me. Thank you Mamun for guiding me at the beginning with many things, always listening, and being a great friend. Heikki, special thanks for your scientific inputs to my projects, especially with Western

blotting. Ashwini, we wrote our PhD thesis at the same time and I am happy that we both made it and finished our studies finally. I would like to thank you and Disha for your support and friendship. Jarno, thank you for all your encouragements, support and positive energy. I would like to especially thank you for proof-reading my thesis. Dimitrios, thank you for cheering me up with nice and lively discussions. In addition, I would like to thank all the current and previous members of the Heckman group: Alun, Minna, Siv, Joseph, Alina, Aino-Maija, Juho, Romika, Amanda, Deb, Shivanshi, Sadiksha, Cristina, Lucie, Simo, Komal, Minxia, Sofia, Annukka, and Samuli for being such nice colleagues.

This journey wouldn't have been the same without the support of many friends. I would like to thank Tea for all the wonderful times we shared together at FIMM and outside. Whether it was having a coffee, shopping or attending a conference, you were there to share the laughs and struggles with me. I am very grateful for your friendship and all those moments. I would like to express my gratitude to dear friends Henna, Maiju, Teija, Pia and other 'biochemistry girls' for your friendship and fun times since our studies. Thank you, Rita, for being such a great friend. I greatly value your support, also with writing this thesis. It has meant a lot to me to have a friend who I could always count on. Thank you, Susanne, Pauliina, Elsa, Anna-Brita, and Alexandra for the nice girls' evenings. In addition, I would like to express my gratitude to friends in Helsinki and abroad for your encouragement and happy moments together: Jessica and Juha, Julio, Evelyn, Evelyn G, Halley and other Latin friends.

Finally, I would like to thank my family and relatives for your love and care. First, I want to express my gratitude to my uncles and cousins. Thank you for all the great cousins' gatherings ☺ Thank you, Elina, for our lovely breakfast meetings over the years. I am grateful to my dear grandmother, Ida for your encouragement and caring. Finally, I am able to tell you that I am done with my studies. Lastly, I want to thank with all my heart my parents, Pirjo and Juhani, my brother Jarkko and his spouse Sini and my dear niece Senja. Thank you for always being there, encouraging and supporting me. You have always reminded me of the things that matter the most.

Helsinki, August 2019

Riikka Karjalainen

9 REFERENCES

1. Cancer Genome Atlas Research N, Ley TJ, Miller C, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med.* 2013;368(22):2059-2074.
2. Lawrence MS, Stojanov P, Mermel CH, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature.* 2014;505(7484):495-501.
3. Short NJ, Rytting ME, Cortes JE. Acute myeloid leukaemia. *Lancet.* 2018;392(10147):593-606.
4. Andreeff M, Konopleva M. Mechanisms of drug resistance in AML. *Cancer Treat Res.* 2002;112:237-262.
5. Tabe Y, Konopleva M. Advances in understanding the leukaemia microenvironment. *British journal of haematology.* 2014;164(6):767-778.
6. Ashkenazi A, Fairbrother WJ, Leverson JD, Souers AJ. From basic apoptosis discoveries to advanced selective BCL-2 family inhibitors. *Nature reviews Drug discovery.* 2017;16(4):273-284.
7. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. *Blood.* 1993;81(11):2844-2853.
8. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. *Cell stem cell.* 2012;10(2):120-136.
9. Till JE, Mc CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.* 1961;14:213-222.
10. Siminovitch L, McCulloch EA, Till JE. The Distribution of Colony-Forming Cells among Spleen Colonies. *J Cell Comp Physiol.* 1963;62:327-336.
11. Siminovitch L, Till JE, McCulloch EA. Decline in Colony-Forming Ability of Marrow Cells Subjected to Serial Transplantation into Irradiated Mice. *J Cell Comp Physiol.* 1964;64:23-31.
12. Wu AM, Till JE, Siminovitch L, McCulloch EA. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol.* 1967;69(2):177-184.
13. Orkin SH. Development of the hematopoietic system. *Current opinion in genetics & development.* 1996;6(5):597-602.
14. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature.* 2000;404(6774):193-197.
15. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 1997;91(5):661-672.
16. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet.* 2000;1(1):57-64.
17. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell.* 2008;132(4):631-644.

18. Rekhtman N, Radparvar F, Evans T, Skoultchi AI. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev.* 1999;13(11):1398-1411.
19. Tsang AP, Visvader JE, Turner CA, et al. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell.* 1997;90(1):109-119.
20. Tsang AP, Fujiwara Y, Hom DB, Orkin SH. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev.* 1998;12(8):1176-1188.
21. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science.* 1994;265(5178):1573-1577.
22. Nerlov C, McNagny KM, Doderlein G, Kowenz-Leutz E, Graf T. Distinct C/EBP functions are required for eosinophil lineage commitment and maturation. *Genes Dev.* 1998;12(15):2413-2423.
23. Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science.* 1992;258(5083):808-812.
24. Georgopoulos K, Bigby M, Wang JH, et al. The Ikaros gene is required for the development of all lymphoid lineages. *Cell.* 1994;79(1):143-156.
25. Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell.* 1994;79(5):901-912.
26. Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature.* 1999;401(6753):556-562.
27. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells.* 1978;4(1-2):7-25.
28. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature.* 2014;505(7483):327-334.
29. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature.* 2003;425(6960):836-841.
30. Hoggatt J, Kfoury Y, Scadden DT. Hematopoietic Stem Cell Niche in Health and Disease. *Annu Rev Pathol.* 2016;11:555-581.
31. Acar M, Kocherlakota KS, Murphy MM, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature.* 2015;526(7571):126-130.
32. Campbell FR. Ultrastructural studies of transmural migration of blood cells in the bone marrow of rats, mice and guinea pigs. *Am J Anat.* 1972;135(4):521-535.
33. Levesque JP, Leavesley DI, Niutta S, Vadas M, Simmons PJ. Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *The Journal of experimental medicine.* 1995;181(5):1805-1815.
34. Qian H, Tryggvason K, Jacobsen SE, Ekblom M. Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone

marrow and collaboration with alpha4 integrins. *Blood.* 2006;107(9):3503-3510.
35. Schmits R, Filmus J, Gerwin N, et al. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood.* 1997;90(6):2217-2233.
36. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc Natl Acad Sci U S A.* 1998;95(24):14423-14428.
37. Katayama Y, Battista M, Kao WM, et al. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell.* 2006;124(2):407-421.
38. Ponomaryov T, Peled A, Petit I, et al. Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *The Journal of clinical investigation.* 2000;106(11):1331-1339.
39. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell.* 2004;118(2):149-161.
40. Nilsson SK, Johnston HM, Whitty GA, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood.* 2005;106(4):1232-1239.
41. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature.* 2012;481(7382):457-462.
42. Greenbaum A, Hsu YM, Day RB, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature.* 2013;495(7440):227-230.
43. Mendez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature.* 2008;452(7186):442-447.
44. Yamazaki S, Ema H, Karlsson G, et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell.* 2011;147(5):1146-1158.
45. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature.* 2013;495(7440):231-235.
46. Clark SC, Kamen R. The human hematopoietic colony-stimulating factors. *Science.* 1987;236(4806):1229-1237.
47. Metcalf D. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature.* 1989;339(6219):27-30.
48. Hoffbrand AV, Moss P.A.H. *Hoffbrand's Essential Haematology.* 7th ed: John Wiley & Sons Ltd; 2016.
49. Stanley ER, Hansen G, Woodcock J, Metcalf D. Colony stimulating factor and the regulation of granulopoiesis and macrophage production. *Fed Proc.* 1975;34(13):2272-2278.

50. Stanley ER, Heard PM. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *The Journal of biological chemistry.* 1977;252(12):4305-4312.
51. Welte K, Gabrilove J, Bronchud MH, Platzer E, Morstyn G. Filgrastim (r-metHuG-CSF): the first 10 years. *Blood.* 1996;88(6):1907-1929.
52. Takei Y AHTK. *Handbook of Hormones Comparative Endocrinology for Basic and Clinical Research.* 2015.
53. Bazan JF. Haemopoietic receptors and helical cytokines. *Immunol Today.* 1990;11(10):350-354.
54. Sieff CA. Hematopoietic growth factors. *The Journal of clinical investigation.* 1987;79(6):1549-1557.
55. Leonard WJ. Role of Jak kinases and STATs in cytokine signal transduction. *Int J Hematol.* 2001;73(3):271-277.
56. Schindler C, Shuai K, Prezioso VR, Darnell JE, Jr. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science.* 1992;257(5071):809-813.
57. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science.* 1994;264(5164):1415-1421.
58. Lord JD, McIntosh BC, Greenberg PD, Nelson BH. The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *J Immunol.* 2000;164(5):2533-2541.
59. Bowman T, Broome MA, Sinibaldi D, et al. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc Natl Acad Sci U S A.* 2001;98(13):7319-7324.
60. Vainchenker W, Constantinescu SN. JAK/STAT signaling in hematological malignancies. *Oncogene.* 2013;32(21):2601-2613.
61. Rodig SJ, Meraz MA, White JM, et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell.* 1998;93(3):373-383.
62. Buckley RH. Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Annu Rev Immunol.* 2004;22:625-655.
63. Yao Z, Cui Y, Watford WT, et al. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A.* 2006;103(4):1000-1005.
64. Xu D, Qu CK. Protein tyrosine phosphatases in the JAK/STAT pathway. *Front Biosci.* 2008;13:4925-4932.
65. Shuai K, Liu B. Regulation of gene-activation pathways by PIAS proteins in the immune system. *Nature reviews Immunology.* 2005;5(8):593-605.
66. Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. *Stem cells.* 2001;19(5):378-387.
67. Pencik J, Pham HT, Schmoellerl J, et al. JAK-STAT signaling in cancer: From cytokines to non-coding genome. *Cytokine.* 2016;87:26-36.

68. Walters DK, Mercher T, Gu TL, et al. Activating alleles of JAK3 in acute megakaryoblastic leukemia. *Cancer cell.* 2006;10(1):65-75.
69. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature.* 2005;434(7037):1144-1148.
70. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* 2005;365(9464):1054-1061.
71. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med.* 2005;352(17):1779-1790.
72. Verstovsek S, Kantarjian H, Mesa RA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med.* 2010;363(12):1117-1127.
73. Domen J, Cheshier SH, Weissman IL. The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. *The Journal of experimental medicine.* 2000;191(2):253-264.
74. Nagata S. Apoptosis by death factor. *Cell.* 1997;88(3):355-365.
75. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* 2007;35(4):495-516.
76. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science.* 1998;281(5381):1305-1308.
77. Leverson JD, Phillips DC, Mitten MJ, et al. Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci Transl Med.* 2015;7(279):279ra240.
78. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science.* 1984;226(4678):1097-1099.
79. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol.* 2014;15(1):49-63.
80. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646-674.
81. Certo M, Del Gaizo Moore V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer cell.* 2006;9(5):351-365.
82. Souers AJ, Leverson JD, Boghaert ER, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nature medicine.* 2013;19(2):202-208.
83. Leverson JD, Sampath D, Souers AJ, et al. Found in Translation: How Preclinical Research Is Guiding the Clinical Development of the BCL2-Selective Inhibitor Venetoclax. *Cancer discovery.* 2017;7(12):1376-1393.

84. Konopleva M, Pollyea DA, Potluri J, et al. Efficacy and Biological Correlates of Response in a Phase II Study of Venetoclax Monotherapy in Patients with Acute Myelogenous Leukemia. *Cancer discovery*. 2016;6(10):1106-1117.
85. Dohner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. *N Engl J Med*. 2015;373(12):1136-1152.
86. National Cancer Institute. Seer Cancer Statistics Fact sheets: Acute Myeloid Leukemia: National Cancer Institute: Bethesda, MD, USA; 2018. https://seer.cancer.gov/statfacts/html/amyl.html.
87. Finnish Cancer Registry. Cancer Statistics 2018. https://cancerregistry.fi/statistics/cancer-statistics/.
88. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 2012;150(2):264-278.
89. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506(7488):328-333.
90. Bowman RL, Busque L, Levine RL. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. *Cell stem cell*. 2018;22(2):157-170.
91. Inoue D, Bradley RK, Abdel-Wahab O. Spliceosomal gene mutations in myelodysplasia: molecular links to clonal abnormalities of hematopoiesis. *Genes Dev*. 2016;30(9):989-1001.
92. Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. 2015;126(1):9-16.
93. Genovese G, Kahler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371(26):2477-2487.
94. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488-2498.
95. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481(7382):506-510.
96. Raaijmakers MH, Mukherjee S, Guo S, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464(7290):852-857.
97. Kode A, Manavalan JS, Mosialou I, et al. Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature*. 2014;506(7487):240-244.
98. Santamaria C, Muntion S, Roson B, et al. Impaired expression of DICER, DROSHA, SBDS and some microRNAs in mesenchymal stromal cells from myelodysplastic syndrome patients. *Haematologica*. 2012;97(8):1218-1224.
99. Wiseman DH. Donor cell leukemia: a review. *Biol Blood Marrow Transplant*. 2011;17(6):771-789.
100. Reichard KK, Zhang QY, Sanchez L, Hozier J, Viswanatha D, Foucar K. Acute myeloid leukemia of donor origin after allogeneic bone marrow

transplantation for precursor T-cell acute lymphoblastic leukemia: case report and review of the literature. *American journal of hematology.* 2006;81(3):178-185.
101. Fiedler W, Graeven U, Ergun S, et al. Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. *Blood.* 1997;89(6):1870-1875.
102. Duarte D, Hawkins ED, Akinduro O, et al. Inhibition of Endosteal Vascular Niche Remodeling Rescues Hematopoietic Stem Cell Loss in AML. *Cell stem cell.* 2018;22(1):64-77 e66.
103. Rowley JD. Chromosomal translocations: revisited yet again. *Blood.* 2008;112(6):2183-2189.
104. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med.* 2016;374(23):2209-2221.
105. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood.* 2016;127(20):2391-2405.
106. Swerdlow SH CE, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J. *World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. Update to 4th Edition.*: IARC; 2017.
107. Podoltsev NA, Stahl M, Zeidan AM, Gore SD. Selecting initial treatment of acute myeloid leukaemia in older adults. *Blood Rev.* 2017;31(2):43-62.
108. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood.* 2017;129(4):424-447.
109. Castaigne S, Pautas C, Terre C, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. *Lancet.* 2012;379(9825):1508-1516.
110. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood.* 2002;100(5):1532-1542.
111. Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *N Engl J Med.* 2017;377(5):454-464.
112. Stone RM, Larson RA, Dohner H. Midostaurin in FLT3-Mutated Acute Myeloid Leukemia. *N Engl J Med.* 2017;377(19):1903.
113. Perl AE, Altman JK, Cortes J, et al. Selective inhibition of FLT3 by gilteritinib in relapsed or refractory acute myeloid leukaemia: a multicentre, first-in-human, open-label, phase 1-2 study. *Lancet Oncol.* 2017;18(8):1061-1075.
114. Lancet JE, Uy GL, Cortes JE, et al. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2018;36(26):2684-2692.

115. DiNardo CD, Pratz K, Pullarkat V, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood.* 2019;133(1):7-17.
116. Mason KD, Carpinelli MR, Fletcher JI, et al. Programmed anuclear cell death delimits platelet life span. *Cell.* 2007;128(6):1173-1186.
117. DiNardo CD, Pratz KW, Letai A, et al. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol.* 2018;19(2):216-228.
118. Metzeler KH, Herold T, Rothenberg-Thurley M, et al. Spectrum and prognostic relevance of driver gene mutations in acute myeloid leukemia. *Blood.* 2016;128(5):686-698.
119. Medeiros BC, Fathi AT, DiNardo CD, Pollyea DA, Chan SM, Swords R. Isocitrate dehydrogenase mutations in myeloid malignancies. *Leukemia.* 2017;31(2):272-281.
120. Stein EM, DiNardo CD, Pollyea DA, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood.* 2017;130(6):722-731.
121. DiNardo CD, Stein EM, de Botton S, et al. Durable Remissions with Ivosidenib in IDH1-Mutated Relapsed or Refractory AML. *N Engl J Med.* 2018;378(25):2386-2398.
122. Schlenk RF, Muller-Tidow C, Benner A, Kieser M. Relapsed/refractory acute myeloid leukemia: any progress? *Curr Opin Oncol.* 2017;29(6):467-473.
123. Schlenk RF, Frech P, Weber D, et al. Impact of pretreatment characteristics and salvage strategy on outcome in patients with relapsed acute myeloid leukemia. *Leukemia.* 2017;31(5):1217-1220.
124. Dombret H, Gardin C. An update of current treatments for adult acute myeloid leukemia. *Blood.* 2016;127(1):53-61.
125. Hammerlindl H, Schaider H. Tumor cell-intrinsic phenotypic plasticity facilitates adaptive cellular reprogramming driving acquired drug resistance. *J Cell Commun Signal.* 2018;12(1):133-141.
126. Gottesman MM. Mechanisms of cancer drug resistance. *Annu Rev Med.* 2002;53:615-627.
127. Bardenheuer W, Lehmberg K, Rattmann I, et al. Resistance to cytarabine and gemcitabine and in vitro selection of transduced cells after retroviral expression of cytidine deaminase in human hematopoietic progenitor cells. *Leukemia.* 2005;19(12):2281-2288.
128. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nature reviews Cancer.* 2002;2(1):48-58.
129. Yang K, Fu LW. Mechanisms of resistance to BCR-ABL TKIs and the therapeutic strategies: A review. *Crit Rev Oncol Hematol.* 2015;93(3):277-292.
130. Tahir SK, Smith ML, Hessler P, et al. Potential mechanisms of resistance to venetoclax and strategies to circumvent it. *BMC cancer.* 2017;17(1):399.

131. Fresquet V, Rieger M, Carolis C, Garcia-Barchino MJ, Martinez-Climent JA. Acquired mutations in BCL2 family proteins conferring resistance to the BH3 mimetic ABT-199 in lymphoma. *Blood.* 2014;123(26):4111-4119.
132. Wang A, Zhong H. Roles of the bone marrow niche in hematopoiesis, leukemogenesis, and chemotherapy resistance in acute myeloid leukemia. *Hematology.* 2018;23(10):729-739.
133. Zeng Z, Samudio IJ, Munsell M, et al. Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias. *Molecular cancer therapeutics.* 2006;5(12):3113-3121.
134. Sison EA, McIntyre E, Magoon D, Brown P. Dynamic chemotherapy-induced upregulation of CXCR4 expression: a mechanism of therapeutic resistance in pediatric AML. *Mol Cancer Res.* 2013;11(9):1004-1016.
135. Zeng Z, Shi YX, Samudio IJ, et al. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood.* 2009;113(24):6215-6224.
136. Traer E, Martinez J, Javidi-Sharifi N, et al. FGF2 from Marrow Microenvironment Promotes Resistance to FLT3 Inhibitors in Acute Myeloid Leukemia. *Cancer research.* 2016;76(22):6471-6482.
137. Parmar A, Marz S, Rushton S, et al. Stromal niche cells protect early leukemic FLT3-ITD+ progenitor cells against first-generation FLT3 tyrosine kinase inhibitors. *Cancer research.* 2011;71(13):4696-4706.
138. Kojima K, McQueen T, Chen Y, et al. p53 activation of mesenchymal stromal cells partially abrogates microenvironment-mediated resistance to FLT3 inhibition in AML through HIF-1alpha-mediated down-regulation of CXCL12. *Blood.* 2011;118(16):4431-4439.
139. Matsunaga T, Takemoto N, Sato T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nature medicine.* 2003;9(9):1158-1165.
140. Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature medicine.* 2006;12(10):1167-1174.
141. Jacamo R, Chen Y, Wang Z, et al. Reciprocal leukemia-stroma VCAM-1/VLA-4-dependent activation of NF-kappaB mediates chemoresistance. *Blood.* 2014;123(17):2691-2702.
142. Behrmann L, Wellbrock J, Fiedler W. Acute Myeloid Leukemia and the Bone Marrow Niche-Take a Closer Look. *Front Oncol.* 2018;8:444.
143. Gordon PM, Dias S, Williams DA. Cytokines secreted by bone marrow stromal cells protect c-KIT mutant AML cells from c-KIT inhibitor-induced apoptosis. *Leukemia.* 2014;28(11):2257-2260.
144. Quintarelli C, De Angelis B, Errichiello S, et al. Selective strong synergism of Ruxolitinib and second generation tyrosine kinase inhibitors to overcome bone marrow stroma related drug resistance in chronic myelogenous leukemia. *Leukemia research.* 2014;38(2):236-242.

145. Sung PJ, Sugita M, Koblish H, Perl AE, Carroll M. Hematopoietic cytokines mediate resistance to targeted therapy in FLT3-ITD acute myeloid leukemia. *Blood Adv.* 2019;3(7):1061-1072.
146. Zhang W, Borthakur G, Gao C, et al. The Dual MEK/FLT3 Inhibitor E6201 Exerts Cytotoxic Activity against Acute Myeloid Leukemia Cells Harboring Resistance-Conferring FLT3 Mutations. *Cancer research.* 2016;76(6):1528-1537.
147. Brandts CH, Sargin B, Rode M, et al. Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer research.* 2005;65(21):9643-9650.
148. Lindblad O, Cordero E, Puissant A, et al. Aberrant activation of the PI3K/mTOR pathway promotes resistance to sorafenib in AML. *Oncogene.* 2016;35(39):5119-5131.
149. Garcia-Manero G, Chuah C, Wilding G, et al. Phase I study of the oral histone deacetylase inhibitor SB939 in patients with advanced hematologic malignancies. In: Am Soc Hematology; 2010.
150. Roboz GJ, Kantarjian HM, Yee KWL, et al. Dose, schedule, safety, and efficacy of guadecitabine in relapsed or refractory acute myeloid leukemia. *Cancer.* 2018;124(2):325-334.
151. Kantarjian HM, Roboz GJ, Kropf PL, et al. Guadecitabine (SGI-110) in treatment-naive patients with acute myeloid leukaemia: phase 2 results from a multicentre, randomised, phase 1/2 trial. *Lancet Oncol.* 2017;18(10):1317-1326.
152. Dawson MA, Prinjha RK, Dittmann A, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature.* 2011;478(7370):529-533.
153. Berthon C, Raffoux E, Thomas X, et al. Bromodomain inhibitor OTX015 in patients with acute leukaemia: a dose-escalation, phase 1 study. *Lancet Haematol.* 2016;3(4):e186-195.
154. Daver N, Schlenk RF, Russell NH, Levis MJ. Targeting FLT3 mutations in AML: review of current knowledge and evidence. *Leukemia.* 2019;33(2):299-312.
155. Paschka P, Schlenk RF, Weber D, et al. Adding dasatinib to intensive treatment in core-binding factor acute myeloid leukemia-results of the AMLSG 11-08 trial. *Leukemia.* 2018;32(7):1621-1630.
156. Gao X, Lin J, Gao L, et al. High expression of c-kit mRNA predicts unfavorable outcome in adult patients with t(8;21) acute myeloid leukemia. *PloS one.* 2015;10(4):e0124241.
157. Lichtenegger FS, Krupka C, Haubner S, Kohnke T, Subklewe M. Recent developments in immunotherapy of acute myeloid leukemia. *J Hematol Oncol.* 2017;10(1):142.
158. Peters C, Brown S. Antibody-drug conjugates as novel anti-cancer chemotherapeutics. *Biosci Rep.* 2015;35(4).
159. Loke J, Khan JN, Wilson JS, Craddock C, Wheatley K. Mylotarg has potent anti-leukaemic effect: a systematic review and meta-analysis of

anti-CD33 antibody treatment in acute myeloid leukaemia. *Annals of hematology.* 2015;94(3):361-373.
160. Krupka C, Kufer P, Kischel R, et al. CD33 target validation and sustained depletion of AML blasts in long-term cultures by the bispecific T-cell-engaging antibody AMG 330. *Blood.* 2014;123(3):356-365.
161. Aigner M, Feulner J, Schaffer S, et al. T lymphocytes can be effectively recruited for ex vivo and in vivo lysis of AML blasts by a novel CD33/CD3-bispecific BiTE antibody construct. *Leukemia.* 2013;27(5):1107-1115.
162. Testa U, Pelosi E, Frankel A. CD 123 is a membrane biomarker and a therapeutic target in hematologic malignancies. *Biomark Res.* 2014;2(1):4.
163. Alatrash G, Daver N, Mittendorf EA. Targeting Immune Checkpoints in Hematologic Malignancies. *Pharmacological reviews.* 2016;68(4):1014-1025.
164. Berger R, Rotem-Yehudar R, Slama G, et al. Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2008;14(10):3044-3051.
165. Konoplev S, Rassidakis GZ, Estey E, et al. Overexpression of CXCR4 predicts adverse overall and event-free survival in patients with unmutated FLT3 acute myeloid leukemia with normal karyotype. *Cancer.* 2007;109(6):1152-1156.
166. Rombouts EJ, Pavic B, Lowenberg B, Ploemacher RE. Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. *Blood.* 2004;104(2):550-557.
167. DiPersio JF, Micallef IN, Stiff PJ, et al. Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2009;27(28):4767-4773.
168. DiPersio JF, Stadtmauer EA, Nademanee A, et al. Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. *Blood.* 2009;113(23):5720-5726.
169. Michelis FV, Hedley DW, Malhotra S, et al. Mobilization of Leukemic Cells Using Plerixafor as Part of a Myeloablative Preparative Regimen for Patients with Acute Myelogenous Leukemia Undergoing Allografting: Assessment of Safety and Tolerability. *Biol Blood Marrow Transplant.* 2019;25(6):1158-1163.
170. Uy GL, Rettig MP, Stone RM, et al. A phase 1/2 study of chemosensitization with plerixafor plus G-CSF in relapsed or refractory acute myeloid leukemia. *Blood Cancer J.* 2017;7(3):e542.

171. Roboz GJ, Ritchie EK, Dault Y, et al. Phase I trial of plerixafor combined with decitabine in newly diagnosed older patients with acute myeloid leukemia. *Haematologica.* 2018;103(8):1308-1316.
172. Natoni A, Macauley MS, O'Dwyer ME. Targeting Selectins and Their Ligands in Cancer. *Front Oncol.* 2016;6:93.
173. Winkler IG, Barbier V, Nowlan B, et al. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nature medicine.* 2012;18(11):1651-1657.
174. DeAngelo DJ, Liesveld JL, Jonas BA, et al. A Phase I/II Study of GMI-1271, a Novel E-Selectin Antagonist, in Combination with Induction Chemotherapy in Relapsed/Refractory and Elderly Previously Untreated Acute Myeloid Leukemia; Results to Date. In: Am Soc Hematology; 2016.
175. DeAngelo DJ, Jonas BA, Liesveld J, et al. GMI-1271, a novel E-selectin antagonist, in combination with chemotherapy in relapsed/refractory AML. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2017;35.
176. Unger FT, Witte I, David KA. Prediction of individual response to anticancer therapy: historical and future perspectives. *Cell Mol Life Sci.* 2015;72(4):729-757.
177. Blom K, Nygren P, Larsson R, Andersson CR. Predictive Value of Ex Vivo Chemosensitivity Assays for Individualized Cancer Chemotherapy: A Meta-Analysis. *SLAS Technol.* 2017;22(3):306-314.
178. Snijder B, Vladimer GI, Krall N, et al. Image-based ex-vivo drug screening for patients with aggressive haematological malignancies: interim results from a single-arm, open-label, pilot study. *Lancet Haematol.* 2017;4(12):e595-e606.
179. Halliwell B. Cell culture, oxidative stress, and antioxidants: avoiding pitfalls. *Biomed J.* 2014;37(3):99-105.
180. Pemovska T, Kontro M, Yadav B, et al. Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. *Cancer discovery.* 2013;3(12):1416-1429.
181. Pemovska T, Johnson E, Kontro M, et al. Axitinib effectively inhibits BCR-ABL1(T315I) with a distinct binding conformation. *Nature.* 2015;519(7541):102-105.
182. Foss EJ, Radulovic D, Stirewalt DL, et al. Proteomic classification of acute leukemias by alignment-based quantitation of LC-MS/MS data sets. *J Proteome Res.* 2012;11(10):5005-5010.
183. Yadav B, Pemovska T, Szwajda A, et al. Quantitative scoring of differential drug sensitivity for individually optimized anticancer therapies. *Scientific reports.* 2014;4:5193.
184. Yadav B, Wennerberg K, Aittokallio T, Tang J. Searching for Drug Synergy in Complex Dose-Response Landscapes Using an Interaction Potency Model. *Comput Struct Biotechnol J.* 2015;13:504-513.
185. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43(7):e47.

186. Kumar A, Kankainen M, Parsons A, Kallioniemi O, Mattila P, Heckman CA. The impact of RNA sequence library construction protocols on transcriptomic profiling of leukemia. *BMC Genomics.* 2017;18(1):629.
187. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114-2120.
188. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013;29(1):15-21.
189. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res.* 2013;41(10):e108.
190. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome biology.* 2010;11(3):R25.
191. Roecklein BA, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood.* 1995;85(4):997-1005.
192. Bendall LJ, Daniel A, Kortlepel K, Gottlieb DJ. Bone marrow adherent layers inhibit apoptosis of acute myeloid leukemia cells. *Exp Hematol.* 1994;22(13):1252-1260.
193. Garrido SM, Appelbaum FR, Willman CL, Banker DE. Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5). *Exp Hematol.* 2001;29(4):448-457.
194. Ward AC, Touw I, Yoshimura A. The Jak-Stat pathway in normal and perturbed hematopoiesis. *Blood.* 2000;95(1):19-29.
195. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene.* 2002;285(1-2):1-24.
196. Kontro M, Kumar A, Majumder MM, et al. HOX gene expression predicts response to BCL-2 inhibition in acute myeloid leukemia. *Leukemia.* 2017;31(2):301-309.
197. Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *The Journal of biological chemistry.* 1991;266(12):7706-7713.
198. Simard JC, Cesaro A, Chapeton-Montes J, et al. S100A8 and S100A9 induce cytokine expression and regulate the NLRP3 inflammasome via ROS-dependent activation of NF-kappaB(1.). *PloS one.* 2013;8(8):e72138.
199. Vogl T, Tenbrock K, Ludwig S, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature medicine.* 2007;13(9):1042-1049.
200. Ortiz ML, Lu L, Ramachandran I, Gabrilovich DI. Myeloid-derived suppressor cells in the development of lung cancer. *Cancer Immunol Res.* 2014;2(1):50-58.

201. Ichikawa M, Williams R, Wang L, Vogl T, Srikrishna G. S100A8/A9 activate key genes and pathways in colon tumor progression. *Mol Cancer Res.* 2011;9(2):133-148.
202. Hibino T, Sakaguchi M, Miyamoto S, et al. S100A9 is a novel ligand of EMMPRIN that promotes melanoma metastasis. *Cancer research.* 2013;73(1):172-183.
203. Grebhardt S, Muller-Decker K, Bestvater F, Hershfinkel M, Mayer D. Impact of S100A8/A9 expression on prostate cancer progression in vitro and in vivo. *J Cell Physiol.* 2014;229(5):661-671.
204. Nicolas E, Ramus C, Berthier S, et al. Expression of S100A8 in leukemic cells predicts poor survival in de novo AML patients. *Leukemia.* 2011;25(1):57-65.
205. Kawasaki H, Nakayama S, Kretsinger RH. Classification and evolution of EF-hand proteins. *Biometals.* 1998;11(4):277-295.
206. Carafoli E. Calcium signaling: a tale for all seasons. *Proc Natl Acad Sci U S A.* 2002;99(3):1115-1122.
207. Szalai G, Krishnamurthy R, Hajnoczky G. Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J.* 1999;18(22):6349-6361.
208. Spijkers-Hagelstein JA, Schneider P, Hulleman E, et al. Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Leukemia.* 2012;26(6):1255-1265.
209. Elo LL, Karjalainen R, Ohman T, et al. Statistical detection of quantitative protein biomarkers provides insights into signaling networks deregulated in acute myeloid leukemia. *Proteomics.* 2014;14(21-22):2443-2453.
210. Lam SS, He AB, Leung AY. Treatment of acute myeloid leukemia in the next decade - Towards real-time functional testing and personalized medicine. *Blood Rev.* 2017;31(6):418-425.
211. Bruserud O, Gjertsen BT, Foss B, Huang TS. New strategies in the treatment of acute myelogenous leukemia (AML): in vitro culture of aml cells--the present use in experimental studies and the possible importance for future therapeutic approaches. *Stem cells.* 2001;19(1):1-11.
212. McMillin DW, Negri JM, Mitsiades CS. The role of tumour-stromal interactions in modifying drug response: challenges and opportunities. *Nature reviews Drug discovery.* 2013;12(3):217-228.
213. Bennett TA, Montesinos P, Moscardo F, et al. Pharmacological profiles of acute myeloid leukemia treatments in patient samples by automated flow cytometry: a bridge to individualized medicine. *Clin Lymphoma Myeloma Leuk.* 2014;14(4):305-318.
214. Knapper S. The clinical development of FLT3 inhibitors in acute myeloid leukemia. *Expert Opin Investig Drugs.* 2011;20(10):1377-1395.
215. Garcia JS, Stone RM. The Development of FLT3 Inhibitors in Acute Myeloid Leukemia. *Hematol Oncol Clin North Am.* 2017;31(4):663-680.
216. Schnittger S, Schoch C, Dugas M, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and

usefulness as a marker for the detection of minimal residual disease. *Blood.* 2002;100(1):59-66.
217. Yang X, Sexauer A, Levis M. Bone marrow stroma-mediated resistance to FLT3 inhibitors in FLT3-ITD AML is mediated by persistent activation of extracellular regulated kinase. *British journal of haematology.* 2014;164(1):61-72.
218. Weisberg E, Liu Q, Nelson E, et al. Using combination therapy to override stromal-mediated chemoresistance in mutant FLT3-positive AML: synergism between FLT3 inhibitors, dasatinib/multi-targeted inhibitors and JAK inhibitors. *Leukemia.* 2012;26(10):2233-2244.
219. Nelson EA, Walker SR, Xiang M, et al. The STAT5 Inhibitor Pimozide Displays Efficacy in Models of Acute Myelogenous Leukemia Driven by FLT3 Mutations. *Genes Cancer.* 2012;3(7-8):503-511.
220. Rosnet O, Buhring HJ, deLapeyriere O, et al. Expression and signal transduction of the FLT3 tyrosine kinase receptor. *Acta Haematol.* 1996;95(3-4):218-223.
221. Song T, Chai G, Liu Y, Yu X, Wang Z, Zhang Z. Bcl-2 phosphorylation confers resistance on chronic lymphocytic leukaemia cells to the BH3 mimetics ABT-737, ABT-263 and ABT-199 by impeding direct binding. *Br J Pharmacol.* 2016;173(3):471-483.
222. Pan R, Hogdal LJ, Benito JM, et al. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. *Cancer discovery.* 2014;4(3):362-375.
223. Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M, Estrov Z. Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces antiapoptotic and proapoptotic signals in acute myeloid leukemia. *Blood.* 2003;102(2):630-637.
224. Puthier D, Derenne S, Barille S, et al. Mcl-1 and Bcl-xL are co-regulated by IL-6 in human myeloma cells. *British journal of haematology.* 1999;107(2):392-395.
225. Gupta VA, Matulis SM, Conage-Pough JE, et al. Bone marrow microenvironment-derived signals induce Mcl-1 dependence in multiple myeloma. *Blood.* 2017;129(14):1969-1979.
226. Kurtz SE, Eide CA, Kaempf A, et al. Molecularly targeted drug combinations demonstrate selective effectiveness for myeloid- and lymphoid-derived hematologic malignancies. *Proc Natl Acad Sci U S A.* 2017;114(36):E7554-E7563.
227. Kurtz SE, Eide CA, Kaempf A, et al. Dual inhibition of JAK1/2 kinases and BCL2: a promising therapeutic strategy for acute myeloid leukemia. *Leukemia.* 2018;32(9):2025-2028.
228. Jung J, Seol HS, Chang S. The Generation and Application of Patient-Derived Xenograft Model for Cancer Research. *Cancer Res Treat.* 2018;50(1):1-10.
229. Yada E, Wada S, Yoshida S, Sasada T. Use of patient-derived xenograft mouse models in cancer research and treatment. *Future Sci OA.* 2018;4(3):FSO271.

230. Hidalgo M, Amant F, Biankin AV, et al. Patient-derived xenograft models: an emerging platform for translational cancer research. *Cancer discovery.* 2014;4(9):998-1013.
231. Du Y, Deng W, Wang Z, et al. Differential subnetwork of chemokines/cytokines in human, mouse, and rat brain cells after oxygen-glucose deprivation. *J Cereb Blood Flow Metab.* 2017;37(4):1425-1434.
232. Chan SM, Thomas D, Corces-Zimmerman MR, et al. Isocitrate dehydrogenase 1 and 2 mutations induce BCL-2 dependence in acute myeloid leukemia. *Nature medicine.* 2015;21(2):178-184.
233. Chyla B, Daver N, Doyle K, et al. Genetic Biomarkers Of Sensitivity and Resistance to Venetoclax Monotherapy in Patients With Relapsed Acute Myeloid Leukemia. *American journal of hematology.* 2018.
234. Zhang H, Wilmot B, Bottomly D, et al. Biomarkers Predicting Venetoclax Sensitivity and Strategies for Venetoclax Combination Treatment. *Blood.* 2018;132(Suppl 1):175-175.
235. Rasmussen KD, Jia G, Johansen JV, et al. Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis. *Genes Dev.* 2015;29(9):910-922.
236. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. *N Engl J Med.* 2017;377(2):111-121.
237. Cull AH, Snetsinger B, Buckstein R, Wells RA, Rauh MJ. Tet2 restrains inflammatory gene expression in macrophages. *Exp Hematol.* 2017;55:56-70 e13.
238. Foell D, Frosch M, Sorg C, Roth J. Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clin Chim Acta.* 2004;344(1-2):37-51.
239. Roth J, Vogl T, Sorg C, Sunderkotter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol.* 2003;24(4):155-158.
240. Stephen E Kurtz CAE, Narain P. Dubey, Andy Kaempf, Shannon K. McWeeney, Cristina E. Tognon, Motomi Mori, Brian J. Druker, Jeffrey W. Tyner and Anupriya Agarwal. Combining p38MAPK Inhibitors with a Second Targeted Agent Enhances Blockade of Inflammatory Signaling-Mediated Survival in Acute Myeloid Leukemia Cells. *Blood.* 2018;132(Suppl 1):2726.
241. Tyner JW, Tognon CE, Bottomly D, et al. Functional genomic landscape of acute myeloid leukaemia. *Nature.* 2018;562(7728):526-531.
242. Peirs S, Frismantas V, Matthijssens F, et al. Targeting BET proteins improves the therapeutic efficacy of BCL-2 inhibition in T-cell acute lymphoblastic leukemia. *Leukemia.* 2017;31(10):2037-2047.
243. Johnson-Farley N, Veliz J, Bhagavathi S, Bertino JR. ABT-199, a BH3 mimetic that specifically targets Bcl-2, enhances the antitumor activity of chemotherapy, bortezomib and JQ1 in "double hit" lymphoma cells. *Leuk Lymphoma.* 2015;56(7):2146-2152.

244. Sun B, Shah B, Fiskus W, et al. Synergistic activity of BET protein antagonist-based combinations in mantle cell lymphoma cells sensitive or resistant to ibrutinib. *Blood.* 2015;126(13):1565-1574.
245. Bui MH, Lin X, Albert DH, et al. Preclinical Characterization of BET Family Bromodomain Inhibitor ABBV-075 Suggests Combination Therapeutic Strategies. *Cancer research.* 2017;77(11):2976-2989.
246. Fiskus W, Cai T, DiNardo CD, et al. Superior efficacy of cotreatment with BET protein inhibitor and BCL2 or MCL1 inhibitor against AML blast progenitor cells. *Blood Cancer J.* 2019;9(2):4.
247. Patel AJ, Liao CP, Chen Z, Liu C, Wang Y, Le LQ. BET bromodomain inhibition triggers apoptosis of NF1-associated malignant peripheral nerve sheath tumors through Bim induction. *Cell Rep.* 2014;6(1):81-92.
248. Li GQ, Guo WZ, Zhang Y, et al. Suppression of BRD4 inhibits human hepatocellular carcinoma by repressing MYC and enhancing BIM expression. *Oncotarget.* 2016;7(3):2462-2474.
249. Aebersold R, Anderson L, Caprioli R, Druker B, Hartwell L, Smith R. Perspective: a program to improve protein biomarker discovery for cancer. *J Proteome Res.* 2005;4(4):1104-1109.
250. Mueller LN, Brusniak MY, Mani DR, Aebersold R. An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. *J Proteome Res.* 2008;7(1):51-61.
251. Hanash S. Progress in mining the human proteome for disease applications. *OMICS.* 2011;15(3):133-139.
252. Listgarten J, Emili A. Statistical and computational methods for comparative proteomic profiling using liquid chromatography-tandem mass spectrometry. *Mol Cell Proteomics.* 2005;4(4):419-434.
253. Oberg AL, Vitek O. Statistical design of quantitative mass spectrometry-based proteomic experiments. *J Proteome Res.* 2009;8(5):2144-2156.
254. Prada-Arismendy J, Arroyave JC, Rothlisberger S. Molecular biomarkers in acute myeloid leukemia. *Blood Rev.* 2017;31(1):63-76.
255. Elo LL, Filen S, Lahesmaa R, Aittokallio T. Reproducibility-optimized test statistic for ranking genes in microarray studies. *IEEE/ACM Trans Comput Biol Bioinform.* 2008;5(3):423-431.
256. Elo LL, Hiissa J, Tuimala J, Kallio A, Korpelainen E, Aittokallio T. Optimized detection of differential expression in global profiling experiments: case studies in clinical transcriptomic and quantitative proteomic datasets. *Brief Bioinform.* 2009;10(5):547-555.
257. Consortium M, Shi L, Reid LH, et al. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nature biotechnology.* 2006;24(9):1151-1161.
258. Lubos E, Loscalzo J, Handy DE. Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal.* 2011;15(7):1957-1997.
259. Pei S, Minhajuddin M, Callahan KP, et al. Targeting aberrant glutathione metabolism to eradicate human acute myelogenous leukemia cells. *The Journal of biological chemistry.* 2013;288(47):33542-33558.